COMPOSITIONS AND METHODS FOR THE THERAPY AND DIAGNOSIS OF BREAST CANCER

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to therapy and diagnosis of cancer, such as breast cancer. The invention is more specifically related to polypeptides, comprising at least a portion of a breast tumor protein, and to polynucleotides encoding such polypeptides. Such polypeptides and polynucleotides are useful in pharmaceutical compositions, *e.g.*, vaccines, and other compositions for the diagnosis and treatment of breast cancer.

BACKGROUND OF THE INVENTION

Field of the Invention

Breast cancer is a significant health problem for women in the United States and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the second leading cause of cancer-related deaths in women, affecting more than 180,000 women in the United States each year. For women in North America, the life-time odds of getting breast cancer are one in eight.

Description of the Related Art

No vaccine or other universally successful method for the prevention or treatment of breast cancer is currently available. Management of the disease currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular breast cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. *See*, *e.g.*, Porter-Jordan and Lippman, *Breast Cancer 8*:73-100 (1994). However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality observed in breast cancer patients

indicates that improvements are needed in the treatment, diagnosis and prevention of the disease.

Accordingly, there is a need in the art for improved methods for the treatment and diagnosis of breast cancer. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides polynucleotide compositions comprising a sequence selected from the group consisting of:

(a) sequences provided in SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576;

(b) complements of the sequences provided in SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576;

(c) sequences consisting of at least 20 contiguous residues of a sequence provided in SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576;

(d) sequences that hybridize to a sequence provided in SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576, under moderately stringent conditions;

(e) sequences having at least 75% identity to a sequence of SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576;

(f) sequences having at least 90% identity to a sequence of SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576; and

(g) degenerate variants of a sequence provided in SEQ ID NO:1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576.

In one preferred embodiment, the polynucleotide compositions of the invention are expressed in at least about 20%, more preferably in at least about 30%, and most preferably in at least about 50% of breast tumors samples tested, at a level that is at least about 2-fold, preferably at least about 5-fold, and most preferably at least about 10-fold higher than that for normal tissues.

The present invention, in another aspect, provides polypeptide compositions comprising an amino acid sequence that is encoded by a polynucleotide sequence described above.

The present invention further provides polypeptide compositions comprising an amino acid sequence selected from the group consisting of sequences recited in SEQ ID NO:62, 176, 179, 181, 469-473, 475, 478, 483, 485, 487, 488, 493-503, 507-509, 514-519, 534-547, 551-553, 565, 570-573, and 577-627.

In certain preferred embodiments, the polypeptides and/or polynucleotides of the present invention are immunogenic, *i.e.*, they are capable of eliciting an immune response, particularly a humoral and/or cellular immune response, as further described herein.

The present invention further provides fragments, variants and/or derivatives of the disclosed polypeptide and/or polynucleotide sequences, wherein the fragments, variants and/or derivatives preferably have a level of immunogenic activity of at least about 50%, preferably at least about 70% and more preferably at least about 90% of the level of immunogenic activity of a polypeptide sequence set forth in SEQ ID NO: 62, 176, 179, 181, 469-473, 475, 478, 483, 485, 487, 488, 493-503, 507-509, 514-519, 534-547, 551-553, 565, 570-573, and 577-627 or a polypeptide sequence encoded by a polynucleotide sequence set forth in SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576.

The present invention further provides polynucleotides that encode a polypeptide described above, expression vectors comprising such polynucleotides and host cells transformed or transfected with such expression vectors.

Within other aspects, the present invention provides pharmaceutical compositions comprising a polypeptide or polynucleotide as described above and a physiologically acceptable carrier.

Within a related aspect of the present invention, the pharmaceutical compositions, *e.g.*, vaccine compositions, are provided for prophylactic or therapeutic applications. Such compositions generally comprise an immunogenic polypeptide or polynucleotide of the invention and an immunostimulant, such as an adjuvant.

The present invention further provides pharmaceutical compositions that comprise: (a) an antibody or antigen-binding fragment thereof that specifically binds to a polypeptide of the present invention, or a fragment thereof; and (b) a physiologically acceptable carrier.

Within further aspects, the present invention provides pharmaceutical compositions comprising: (a) an antigen presenting cell that expresses a polypeptide as described above and (b) a pharmaceutically acceptable carrier or excipient. Illustrative antigen presenting cells include dendritic cells, macrophages, monocytes, fibroblasts and B cells.

Within related aspects, pharmaceutical compositions are provided that comprise: (a) an antigen presenting cell that expresses a polypeptide as described above and (b) an immunostimulant.

The present invention further provides, in other aspects, fusion proteins that comprise at least one polypeptide as described above, as well as polynucleotides encoding such fusion proteins, typically in the form of pharmaceutical compositions, *e.g.*, vaccine compositions, comprising a physiologically acceptable carrier and/or an immunostimulant. The fusions proteins may comprise multiple immunogenic polypeptides or portions/variants thereof, as described herein, and may further comprise one or more polypeptide segments for facilitating the expression, purification and/or immunogenicity of the polypeptide(s).

The present invention further provides, in other aspects, fusion proteins that comprise at least one polypeptide as described above, as well as polynucleotides encoding such fusion proteins. Exemplary fusion proteins according to the present invention comprise a first amino acid portion and a second amino acid portion wherein the first amino acid portion includes 9 or more contiguous amino acids from mammaglobin as depicted by amino acids 1-93 of SEQ ID NO:493 (SEQ ID NO:503); wherein the second amino acid portion includes 9 or more contiguous amino acids from B726P as depicted by SEQ ID NO:475, SEQ ID NO:469, or SEQ ID NO:176; and wherein the first amino acid portion is connected to either the amino terminal or carboxy-terminal end of the second amino acid portion.

Still further embodiments of the present invention provide fusion proteins wherein said first amino acid portion is selected from the group consisting of: IDELKECFLNQTDETLSNVE (SEQ ID NO:496; amino acids 59-78 of SEQ ID NO:493); TTNAIDELKECFLNQ (SEQ ID NO:497; amino acids 55-69 of SEQ ID NO:493); SQHCYAGSGCPLLENVISKTI (SEQ ID NO:498; amino acids 13-33 of SEQ ID NO:493); EYKELLQEFIDDNATTNAID (SEQ ID NO:499; amino acids 41-60 of SEQ ID NO:493); KLLMVLMLA (SEQ ID NO:500; amino acids 2-10 of SEQ ID NO:493); QEFIDDNATTNAI (SEQ ID NO:501; amino acids 47-59 of SEQ ID NO:493); LKECFLNQTDETL (SEQ ID NO:502; amino acids 62-74 of SEQ ID NO:493), and any one of the amino acid sequences set forth in SEQ ID NO:578-593.

Alternative embodiments provide fusion proteins wherein the second amino acid portion includes 9 or more contiguous amino acids encoded by (1) the combined upstream and downstream open reading frame (ORF) of B726P as depicted in SEQ ID NO:475; (2) the upstream ORF of B726P as depicted in SEQ ID NO:469; and (3) the downstream ORF of B726P as depicted in SEQ ID NO:176. Fusion proteins according to the present invention may also comprise a second amino acid portion that includes 9 or more contiguous amino acids from the amino acid sequence depicted by amino acids 1-129 of SEQ ID NO:475. Still additional exemplary fusion proteins are depicted herein by SEQ ID NO:493, SEQ ID NO:494, and SEQ ID NO:495.

Fusion proteins are provided wherein the mammaglobin amino acid portion is connected to the amino-terminus of the B726P amino acid portion while other fusion proteins are provided wherein the mammaglobin amino acid portion is connected to the carboxy-terminus of the B726P amino acid portion. The connection between the mammaglobin amino acid portion and the B726P portion may be a covalent bond. Additionally, a stretch of amino acids either unrelated or related to either mammaglobin and/or B726P may be incorporated between or either amino- or carboxy-terminal to either the mammaglobin and/or B726P amino acid portion.

The present invention also provides isolated polynucleotides that encode any of the fusion proteins that are specifically disclosed herein as well as those fusion proteins that may be accomplished with routine experimentation by the ordinarily skilled artisan.

Within further aspects, the present invention provides methods for stimulating an immune response in a patient, preferably a T cell response in a human patient, comprising administering a pharmaceutical composition described herein. The patient may be afflicted with breast cancer, in which case the methods provide treatment for the disease, or patient considered at risk for such a disease may be treated prophylactically.

Within further aspects, the present invention provides methods for inhibiting the development of a cancer in a patient, comprising administering to a patient a pharmaceutical composition as recited above. The patient may be afflicted with breast cancer, in which case the methods provide treatment for the disease, or patient considered at risk for such a disease may be treated prophylactically.

The present invention further provides, within other aspects, methods for removing tumor cells from a biological sample, comprising contacting a biological sample with T cells that specifically react with a polypeptide of the present invention, wherein the step of contacting is performed under conditions and for a time sufficient to permit the removal of cells expressing the protein from the sample.

Within related aspects, methods are provided for inhibiting the development of a cancer in a patient, comprising administering to a patient a biological sample treated as described above.

Methods are further provided, within other aspects, for stimulating and/or expanding T cells specific for a polypeptide of the present invention, comprising contacting T cells with one or more of: (i) a polypeptide as described above; (ii) a polynucleotide encoding such a polypeptide; and/or (iii) an antigen presenting cell that expresses such a polypeptide; under conditions and for a time sufficient to permit the stimulation and/or expansion of T cells. Isolated T cell populations comprising T cells prepared as described above are also provided.

Within further aspects, the present invention provides methods for inhibiting the development of a cancer in a patient, comprising administering to a patient an effective amount of a T cell population as described above.

The present invention further provides methods for inhibiting the development of a cancer in a patient, comprising the steps of: (a) incubating $CD4^+$ and/or $CD8^+$ T cells isolated from a patient with one or more of: (i) a polypeptide comprising at least an immunogenic portion of polypeptide disclosed herein; (ii) a polynucleotide encoding such a polypeptide; and (iii) an antigen-presenting cell that expressed such a polypeptide; and (b) administering to the patient an effective amount of the proliferated T cells, and thereby inhibiting the development of a cancer in the patient. Proliferated cells may, but need not, be cloned prior to administration to the patient.

Within further aspects, the present invention provides methods for determining the presence or absence of a cancer, preferably a breast cancer, in a patient comprising: (a) contacting a biological sample obtained from a patient with a binding agent that binds to a polypeptide as recited above; (b) detecting in the sample an amount of polypeptide that binds to the binding agent; and (c) comparing the amount of polypeptide with a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient. Within preferred embodiments, the binding agent is an antibody, more preferably a monoclonal antibody.

The present invention also provides, within other aspects, methods for monitoring the progression of a cancer in a patient. Such methods comprise the steps of: (a) contacting a biological sample obtained from a patient at a first point in time with a

binding agent that binds to a polypeptide as recited above; (b) detecting in the sample an amount of polypeptide that binds to the binding agent; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polypeptide detected in step (c) with the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

The present invention further provides, within other aspects, methods for determining the presence or absence of a cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a polypeptide of the present invention; (b) detecting in the sample a level of a polynucleotide, preferably mRNA, that hybridizes to the oligonucleotide; and (c) comparing the level of polynucleotide that hybridizes to the oligonucleotide with a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient. Within certain embodiments, the amount of mRNA is detected via polymerase chain reaction using, for example, at least one oligonucleotide primer that hybridizes to a polynucleotide encoding a polypeptide as recited above, or a complement of such a polynucleotide. Within other embodiments, the amount of mRNA is detected using a hybridization technique, employing an oligonucleotide probe that hybridizes to a polynucleotide that encodes a polypeptide as recited above, or a complement of such a polynucleotide.

In related aspects, methods are provided for monitoring the progression of a cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a polypeptide of the present invention; (b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) with the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

Within further aspects, the present invention provides antibodies, such as monoclonal antibodies, that bind to a polypeptide as described above, as well as diagnostic

kits comprising such antibodies. Diagnostic kits comprising one or more oligonucleotide probes or primers as described above are also provided.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE IDENTIFIERS

Fig. 1 shows the results of a Northern blot of the clone SYN18C6 (SEQ ID NO:40).

Fig. 2 shows the results of an IFN-gamma ELISPOT assay demonstrating that the B726P-specific CTL clone recognizes and lyses breast tumor cell lines expressing B726P.

SEQ ID NO:1 is the determined cDNA sequence of JBT2.

SEQ ID NO:2 is the determined cDNA sequence of JBT6.

SEQ ID NO:3 is the determined cDNA sequence of JBT7.

SEQ ID NO:4 is the determined cDNA sequence of JBT10.

SEQ ID NO:5 is the determined cDNA sequence of JBT13.

SEQ ID NO:6 is the determined cDNA sequence of JBT14.

SEQ ID NO:7 is the determined cDNA sequence of JBT15.

SEQ ID NO:8 is the determined cDNA sequence of JBT16.

SEQ ID NO:9 is the determined cDNA sequence of JBT17.

SEQ ID NO:10 is the determined cDNA sequence of JBT22.

SEQ ID NO:11 is the determined cDNA sequence of JBT25.

SEQ ID NO:12 is the determined cDNA sequence of JBT28.

SEQ ID NO:13 is the determined cDNA sequence of JBT32.

SEQ ID NO:14 is the determined cDNA sequence of JBT33.

SEQ ID NO:15 is the determined cDNA sequence of JBT34.

SEQ ID NO:16 is the determined cDNA sequence of JBT36.

SEQ ID NO:17 is the determined cDNA sequence of JBT37.

SEQ ID NO:18 is the determined cDNA sequence of JBT51.

SEQ ID NO:19 is the determined cDNA sequence of JBTT1.

SEQ ID NO:20 is the determined cDNA sequence of JBTT7.

SEQ ID NO:21 is the determined cDNA sequence of JBTT11.

SEQ ID NO:22 is the determined cDNA sequence of JBTT14.

SEQ ID NO:23 is the determined cDNA sequence of JBTT18.

SEQ ID NO:24 is the determined cDNA sequence of JBTT19.

SEQ ID NO:25 is the determined cDNA sequence of JBTT20.

SEQ ID NO:26 is the determined cDNA sequence of JBTT21.

SEQ ID NO:27 is the determined cDNA sequence of JBTT22.

SEQ ID NO:28 is the determined cDNA sequence of JBTT28.

SEQ ID NO:29 is the determined cDNA sequence of JBTT29.

SEQ ID NO:30 is the determined cDNA sequence of JBTT33.

SEQ ID NO:31 is the determined cDNA sequence of JBTT37.

SEQ ID NO:32 is the determined cDNA sequence of JBTT38.

SEQ ID NO:33 is the determined cDNA sequence of JBTT47.

SEQ ID NO:34 is the determined cDNA sequence of JBTT48.

SEQ ID NO:35 is the determined cDNA sequence of JBTT50.

SEQ ID NO:36 is the determined cDNA sequence of JBTT51.

SEQ ID NO:37 is the determined cDNA sequence of JBTT52.

SEQ ID NO:38 is the determined cDNA sequence of JBTT54.

SEQ ID NO:39 is the determined cDNA sequence of SYN17F4.

SEQ ID NO:40 is the determined cDNA sequence of SYN18C6 (also known as B709P).

SEQ ID NO:41 is the determined cDNA sequence of SYN19A2.

SEQ ID NO:42 is the determined cDNA sequence of SYN19C8.

SEQ ID NO:43 is the determined cDNA sequence of SYN20A12.

SEQ ID NO:44 is the determined cDNA sequence of SYN20G6.

SEQ ID NO:45 is the determined cDNA sequence of SYN20G6-2.

SEQ ID NO:46 is the determined cDNA sequence of SYN21B9.

SEQ ID NO:47 is the determined cDNA sequence of SYN21B9-2.

SEQ ID NO:48 is the determined cDNA sequence of SYN21C10.

SEQ ID NO:49 is the determined cDNA sequence of SYN21G10.

SEQ ID NO:50 is the determined cDNA sequence of SYN21G10-2.

SEQ ID NO:51 is the determined cDNA sequence of SYN21G11.

SEQ ID NO:52 is the determined cDNA sequence of SYN21G11-2.

SEQ ID NO:53 is the determined cDNA sequence of SYN21H8.

SEQ ID NO:54 is the determined cDNA sequence of SYN22A10.

SEQ ID NO:55 is the determined cDNA sequence of SYN22A10-2.

SEQ ID NO:56 is the determined cDNA sequence of SYN22A12.

SEQ ID NO:57 is the determined cDNA sequence of SYN22A2 (also referred to as B718P).

SEQ ID NO:58 is the determined cDNA sequence of SYN22B4.

SEQ ID NO:59 is the determined cDNA sequence of SYN22C2.

SEQ ID NO:60 is the determined cDNA sequence of SYN22E10.

SEQ ID NO:61 is the determined cDNA sequence of SYN22F2.

SEQ ID NO:62 is a predicted amino acid sequence for SYN18C6 (also known as B709P).

SEQ ID NO:63 is the determined cDNA sequence of B723P.

SEQ ID NO:64 is the determined cDNA sequence for B724P.

SEQ ID NO:65 is the determined cDNA sequence of B770P.

SEQ ID NO:66 is the determined cDNA sequence of B716P.

SEQ ID NO:67 is the determined cDNA sequence of B725P.

SEQ ID NO:68 is the determined cDNA sequence of B717P.

SEQ ID NO:69 is the determined cDNA sequence of B771P.

SEQ ID NO:70 is the determined cDNA sequence of B722P.

SEQ ID NO:71 is the determined cDNA sequence of B726P.

SEQ ID NO:72 is the determined cDNA sequence of B727P.

SEQ ID NO:73 is the determined cDNA sequence of B728P.

SEQ ID NO:74-87 are the determined cDNA sequences of isolated clones which show homology to known sequences.

SEQ ID NO:88 is the determined cDNA sequence of 13053.

SEQ ID NO:89 is the determined cDNA sequence of 13057.

SEQ ID NO:90 is the determined cDNA sequence of 13059.

SEQ ID NO:91 is the determined cDNA sequence of 13065.

SEQ ID NO:92 is the determined cDNA sequence of 13067.

SEQ ID NO:93 is the determined cDNA sequence of 13068.

SEQ ID NO:94 is the determined cDNA sequence of 13071.

SEQ ID NO:95 is the determined cDNA sequence of 13072.

SEQ ID NO:96 is the determined cDNA sequence of 13073.

SEQ ID NO:97 is the determined cDNA sequence of 13075.

SEQ ID NO:98 is the determined cDNA sequence of 13078.

SEQ ID NO:99 is the determined cDNA sequence of 13079.

SEQ ID NO:100 is the determined cDNA sequence of 13081.

SEQ ID NO:101 is the determined cDNA sequence of 13082.

SEQ ID NO:102 is the determined cDNA sequence of 13092.

SEQ ID NO:103 is the determined cDNA sequence of 13097.

SEQ ID NO:104 is the determined cDNA sequence of 13101.

SEQ ID NO:105 is the determined cDNA sequence of 13102.

SEQ ID NO:106 is the determined cDNA sequence of 13119.

SEQ ID NO:107 is the determined cDNA sequence of 13131.

SEQ ID NO:108 is the determined cDNA sequence of 13133.

SEQ ID NO:109 is the determined cDNA sequence of 13135.

SEQ ID NO:110 is the determined cDNA sequence of 13139.

SEQ ID NO:111 is the determined cDNA sequence of 13140.

SEQ ID NO:112 is the determined cDNA sequence of 13146.

SEQ ID NO:113 is the determined cDNA sequence of 13147.

SEQ ID NO:114 is the determined cDNA sequence of 13148.

SEQ ID NO:115 is the determined cDNA sequence of 13149.

SEQ ID NO:116 is the determined cDNA sequence of 13151.

SEQ ID NO:117 is the determined cDNA sequence of 13051

SEQ ID NO:118 is the determined cDNA sequence of 13052

SEQ ID NO:119 is the determined cDNA sequence of 13055

SEQ ID NO:120 is the determined cDNA sequence of 13058

SEQ ID NO:121 is the determined cDNA sequence of 13062

SEQ ID NO:122 is the determined cDNA sequence of 13064

SEQ ID NO:123 is the determined cDNA sequence of 13080

SEQ ID NO:124 is the determined cDNA sequence of 13093

SEQ ID NO:125 is the determined cDNA sequence of 13094

SEQ ID NO:126 is the determined cDNA sequence of 13095

SEQ ID NO:127 is the determined cDNA sequence of 13096

SEQ ID NO:128 is the determined cDNA sequence of 13099

SEQ ID NO:129 is the determined cDNA sequence of 13100

SEQ ID NO:130 is the determined cDNA sequence of 13103

SEQ ID NO:131 is the determined cDNA sequence of 13106

SEQ ID NO:132 is the determined cDNA sequence of 13107

SEQ ID NO:133 is the determined cDNA sequence of 13108

SEQ ID NO:134 is the determined cDNA sequence of 13121

SEQ ID NO:135 is the determined cDNA sequence of 13126

SEQ ID NO:136 is the determined cDNA sequence of 13129

SEQ ID NO:137 is the determined cDNA sequence of 13130

SEQ ID NO:138 is the determined cDNA sequence of 13134

SEQ ID NO:139 is the determined cDNA sequence of 13141

SEQ ID NO:140 is the determined cDNA sequence of 13142

SEQ ID NO:141 is the determined cDNA sequence of 14376

SEQ ID NO:142 is the determined cDNA sequence of 14377
SEQ ID NO:143 is the determined cDNA sequence of 14383
SEQ ID NO:144 is the determined cDNA sequence of 14384
SEQ ID NO:145 is the determined cDNA sequence of 14387
SEQ ID NO:146 is the determined cDNA sequence of 14392
SEQ ID NO:147 is the determined cDNA sequence of 14394
SEQ ID NO:148 is the determined cDNA sequence of 14398
SEQ ID NO:149 is the determined cDNA sequence of 14401
SEQ ID NO:150 is the determined cDNA sequence of 14402
SEQ ID NO:151 is the determined cDNA sequence of 14405
SEQ ID NO:152 is the determined cDNA sequence of 14409
SEQ ID NO:153 is the determined cDNA sequence of 14412
SEQ ID NO:154 is the determined cDNA sequence of 14414
SEQ ID NO:155 is the determined cDNA sequence of 14415
SEQ ID NO:156 is the determined cDNA sequence of 14416
SEQ ID NO:157 is the determined cDNA sequence of 14419
SEQ ID NO:158 is the determined cDNA sequence of 14426
SEQ ID NO:159 is the determined cDNA sequence of 14427
SEQ ID NO:160 is the determined cDNA sequence of 14375
SEQ ID NO:161 is the determined cDNA sequence of 14378
SEQ ID NO:162 is the determined cDNA sequence of 14379
SEQ ID NO:163 is the determined cDNA sequence of 14380
SEQ ID NO:164 is the determined cDNA sequence of 14381
SEQ ID NO:165 is the determined cDNA sequence of 14382
SEQ ID NO:166 is the determined cDNA sequence of 14388
SEQ ID NO:167 is the determined cDNA sequence of 14399
SEQ ID NO:168 is the determined cDNA sequence of 14406
SEQ ID NO:169 is the determined cDNA sequence of 14407
SEQ ID NO:170 is the determined cDNA sequence of 14408

SEQ ID NO:171 is the determined cDNA sequence of 14417

SEQ ID NO:172 is the determined cDNA sequence of 14418

SEQ ID NO:173 is the determined cDNA sequence of 14423

SEQ ID NO:174 is the determined cDNA sequence of 14424

SEQ ID NO:175 is the determined cDNA sequence of B726P-20

SEQ ID NO:176 is the predicted amino acid sequence of B726P-20 (also referred to as B726P downstream ORF)

SEQ ID NO:177 is a PCR primer

SEQ ID NO:178 is the determined cDNA sequence of B726P-74

SEQ ID NO:179 is the predicted amino acid sequence of B726P-74

SEQ ID NO:180 is the determined cDNA sequence of B726P-79

SEQ ID NO:181 is the predicted amino acid sequence of B726P-79

SEQ ID NO:182 is the determined cDNA sequence of 19439.1, showing homology to the mammaglobin gene

SEQ ID NO:183 is the determined cDNA sequence of 19407.1, showing homology to the human keratin gene

SEQ ID NO:184 is the determined cDNA sequence of 19428.1, showing homology to human chromosome 17 clone

SEQ ID NO:185 is the determined cDNA sequence of B808P (19408), showing no significant homology to any known gene

SEQ ID NO:186 is the determined cDNA sequence of 19460.1, showing no significant homology to any known gene

SEQ ID NO:187 is the determined cDNA sequence of 19419.1, showing homology to Ig kappa light chain

SEQ ID NO:188 is the determined cDNA sequence of 19411.1, showing homology to human alpha-1 collagen

SEQ ID NO:189 is the determined cDNA sequence of 19420.1, showing homology to mus musculus proteinase-3

SEQ ID NO:190 is the determined cDNA sequence of 19432.1, showing homology to human high motility group box

SEQ ID NO:191 is the determined cDNA sequence of 19412.1, showing homology to the human plasminogen activator gene

SEQ ID NO:192 is the determined cDNA sequence of 19415.1, showing homology to mitogen activated protein kinase

SEQ ID NO:193 is the determined cDNA sequence of 19409.1, showing homology to the chondroitin sulfate proteoglycan protein

SEQ ID NO:194 is the determined cDNA sequence of 19406.1, showing no significant homology to any known gene

SEQ ID NO:195 is the determined cDNA sequence of 19421.1, showing homology to human fibronectin

SEQ ID NO:196 is the determined cDNA sequence of 19426.1, showing homology to the retinoic acid receptor responder 3

SEQ ID NO:197 is the determined cDNA sequence of 19425.1, showing homology to MyD88 mRNA

SEQ ID NO:198 is the determined cDNA sequence of 19424.1, showing homology to peptide transporter (TAP-1) mRNA

SEQ ID NO:199 is the determined cDNA sequence of 19429.1, showing no significant homology to any known gene

SEQ ID NO:200 is the determined cDNA sequence of 19435.1, showing homology to human polymorphic epithelial mucin

SEQ ID NO:201 is the determined cDNA sequence of B813P (19434.1), showing homology to human GATA-3 transcription factor

SEQ ID NO:202 is the determined cDNA sequence of 19461.1, showing homology to the human AP-2 gene

SEQ ID NO:203 is the determined cDNA sequence of 19450.1, showing homology to DNA binding regulatory factor

SEQ ID NO:204 is the determined cDNA sequence of 19451.1, showing homology to Na/H exchange regulatory co-factor

SEQ ID NO:205 is the determined cDNA sequence of 19462.1, showing no significant homology to any known gene

SEQ ID NO:206 is the determined cDNA sequence of 19455.1, showing homology to human mRNA for histone HAS.Z

SEQ ID NO:207 is the determined cDNA sequence of 19459.1, showing homology to PAC clone 179N16

SEQ ID NO:208 is the determined cDNA sequence of 19464.1, showing no significant homology to any known gene

SEQ ID NO:209 is the determined cDNA sequence of 19414.1, showing homology to lipophilin B

SEQ ID NO:210 is the determined cDNA sequence of 19413.1, showing homology to chromosome 17 clone hRPK.209_J_20

SEQ ID NO:211 is the determined cDNA sequence of 19416.1, showing no significant homology to any known gene

SEQ ID NO:212 is the determined cDNA sequence of 19437.1, showing homology to human clone 24976 mRNA

SEQ ID NO:213 is the determined cDNA sequence of 19449.1, showing homology to mouse DNA for PG-M core protein

SEQ ID NO:214 is the determined cDNA sequence of 19446.1, showing no significant homology to any known gene

SEQ ID NO:215 is the determined cDNA sequence of 19452.1, showing no significant homology to any known gene

SEQ ID NO:216 is the determined cDNA sequence of 19483.1, showing no significant homology to any known gene

SEQ ID NO:217 is the determined cDNA sequence of 19526.1, showing homology to human lipophilin C

SEQ ID NO:218 is the determined cDNA sequence of 19484.1, showing homology to the secreted cement gland protein XAG-2

SEQ ID NO:219 is the determined cDNA sequence of 19470.1, showing no significant homology to any known gene

SEQ ID NO:220 is the determined cDNA sequence of 19469.1, showing homology to the human HLA-DM gene

SEQ ID NO:221 is the determined cDNA sequence of 19482.1, showing homology to the human pS2 protein gene

SEQ ID NO:222 is the determined cDNA sequence of B805P (19468.1), showing no significant homology to any known gene

SEQ ID NO:223 is the determined cDNA sequence of 19467.1, showing homology to human thrombospondin mRNA

SEQ ID NO:224 is the determined cDNA sequence of 19498.1, showing homology to the CDC2 gene involved in cell cycle control

SEQ ID NO:225 is the determined cDNA sequence of 19506.1, showing homology to human cDNA for TREB protein

SEQ ID NO:226 is the determined cDNA sequence of B806P (19505.1), showing no significant homology to any known gene

SEQ ID NO:227 is the determined cDNA sequence of 19486.1, showing homology to type I epidermal keratin

SEQ ID NO:228 is the determined cDNA sequence of 19510.1, showing homology to glucose transporter for glycoprotein

SEQ ID NO:229 is the determined cDNA sequence of 19512.1, showing homology to the human lysyl hydroxylase gene

SEQ ID NO:230 is the determined cDNA sequence of 19511.1, showing homology to human palimotoyl-protein thioesterase

SEQ ID NO:231 is the determined cDNA sequence of 19508.1, showing homology to human alpha enolase

SEQ ID NO:232 is the determined cDNA sequence of B807P (19509.1), showing no significant homology to any known gene

SEQ ID NO:233 is the determined cDNA sequence of B809P (19520.1), showing homology to clone 102D24 on chromosome 11q13.31

SEQ ID NO:234 is the determined cDNA sequence of 19507.1, showing homology toprosome beta-subunit

SEQ ID NO:235 is the determined cDNA sequence of 19525.1, showing homology to human pro-urokinase precursor

SEQ ID NO:236 is the determined cDNA sequence of 19513.1, showing no significant homology to any known gene

SEQ ID NO:237 is the determined cDNA sequence of 19517.1, showing homology to human PAC 128M19 clone

SEQ ID NO:238 is the determined cDNA sequence of 19564.1, showing homology to human cytochrome P450-IIB

SEQ ID NO:239 is the determined cDNA sequence of 19553.1, showing homology to human GABA-A receptor pi subunit

SEQ ID NO:240 is the determined cDNA sequence of B811P (19575.1), showing no significant homology to any known gene

SEQ ID NO:241 is the determined cDNA sequence of B810P (19560.1), showing no significant homology to any known gene

SEQ ID NO:242 is the determined cDNA sequence of 19588.1, showing homology to aortic carboxypetidase-like protein

SEQ ID NO:243 is the determined cDNA sequence of 19551.1, showing homology to human BCL-1 gene

SEQ ID NO:244 is the determined cDNA sequence of 19567.1, showing homology to human proteasome-related mRNA

SEQ ID NO:245 is the determined cDNA sequence of B803P (19583.1), showing no significant homology to any known gene

SEQ ID NO:246 is the determined cDNA sequence of B812P (19587.1), showing no significant homology to any known gene

SEQ ID NO:247 is the determined cDNA sequence of B802P (19392.2), showing homology to human chromosome 17

SEQ ID NO:248 is the determined cDNA sequence of 19393.2, showing homology to human nicein B2 chain

SEQ ID NO:249 is the determined cDNA sequence of 19398.2, human MHC class II DQ alpha mRNA

SEQ ID NO:250 is the determined cDNA sequence of B804P (19399.2), showing homology to human Xp22 BAC GSHB-184P14

SEQ ID NO:251 is the determined cDNA sequence of 19401.2, showing homology to human ikB kinase-b gene

SEQ ID NO:252 is the determined cDNA sequence of 20266, showing no significant homology to any known gene

SEQ ID NO:253 is the determined cDNA sequence of B826P (20270), showing no significant homology to any known gene

SEQ ID NO:254 is the determined cDNA sequence of 20274, showing no significant homology to any known gene

SEQ ID NO:255 is the determined cDNA sequence of 20276, showing no significant homology to any known gene

SEQ ID NO:256 is the determined cDNA sequence of 20277, showing no significant homology to any known gene

SEQ ID NO:257 is the determined cDNA sequence of B823P (20280), showing no significant homology to any known gene

SEQ ID NO:258 is the determined cDNA sequence of B821P (20281), showing no significant homology to any known gene

SEQ ID NO:259 is the determined cDNA sequence of B824P (20294), showing no significant homology to any known gene

SEQ ID NO:260 is the determined cDNA sequence of 20303, showing no significant homology to any known gene

SEQ ID NO:261 is the determined cDNA sequence of B820P (20310), showing no significant homology to any known gene

SEQ ID NO:262 is the determined cDNA sequence of B825P (20336), showing no significant homology to any known gene

SEQ ID NO:263 is the determined cDNA sequence of B827P (20341), showing no significant homology to any known gene

SEQ ID NO:264 is the determined cDNA sequence of 20941, showing no significant homology to any known gene

SEQ ID NO:265 is the determined cDNA sequence of 20954, showing no significant homology to any known gene

SEQ ID NO:266 is the determined cDNA sequence of 20961, showing no significant homology to any known gene

SEQ ID NO:267 is the determined cDNA sequence of 20965, showing no significant homology to any known gene

SEQ ID NO:268 is the determined cDNA sequence of 20975, showing no significant homology to any known gene

SEQ ID NO:269 is the determined cDNA sequence of 20261, showing homology to Human p120 catenin

SEQ ID NO:270 is the determined cDNA sequence of B822P (20262), showing homology to Human membrane glycoprotein 4F2

SEQ ID NO:271 is the determined cDNA sequence of 20265, showing homology to Human Na, K-ATPase Alpha 1

SEQ ID NO:272 is the determined cDNA sequence of 20267, showing homology to Human heart HS 90, partial cds

SEQ ID NO:273 is the determined cDNA sequence of 20268, showing homology to Human mRNA GPI-anchored protein p137

SEQ ID NO:274 is the determined cDNA sequence of 20271, showing homology to Human cleavage stimulation factor 77 kDa subunit

SEQ ID NO:275 is the determined cDNA sequence of 20272, showing homology to Human p190-B

SEQ ID NO:276 is the determined cDNA sequence of 20273, showing homology to Human ribophorin

SEQ ID NO:277 is the determined cDNA sequence of 20278, showing homology to Human ornithine amino transferase

SEQ ID NO:278 is the determined cDNA sequence of 20279, showing homology to Human S-adenosylmethionine synthetase

SEQ ID NO:279 is the determined cDNA sequence of 20293, showing homology to Human x inactivation transcript

SEQ ID NO:280 is the determined cDNA sequence of 20300, showing homology to Human cytochrome p450

SEQ ID NO:281 is the determined cDNA sequence of 20305, showing homology to Human elongation factor-1 alpha

SEQ ID NO:282 is the determined cDNA sequence of 20306, showing homology to Human epithelial ets protein

SEQ ID NO:283 is the determined cDNA sequence of 20307, showing homology to Human signal transducer mRNA

SEQ ID NO:284 is the determined cDNA sequence of 20313, showing homology to Human GABA-A receptor pi subunit mRNA

SEQ ID NO:285 is the determined cDNA sequence of 20317, showing homology to Human tyrosine phosphatase

SEQ ID NO:286 is the determined cDNA sequence of 20318, showing homology to Human cathepsine B proteinase

SEQ ID NO:287 is the determined cDNA sequence of 20320, showing homology to Human 2-phosphopyruvate-hydratase-alpha-enolase

SEQ ID NO:288 is the determined cDNA sequence of 20321, showing homology to Human E-cadherin

SEQ ID NO:289 is the determined cDNA sequence of 20322, showing homology to Human hsp86

SEQ ID NO:290 is the determined cDNA sequence of B828P (20326), showing homology to Human x inactivation transcript

SEQ ID NO:291 is the determined cDNA sequence of 20333, showing homology to Human chromatin regulator, SMARCA5

SEQ ID NO:292 is the determined cDNA sequence of 20335, showing homology to Human sphingolipid activator protein 1

SEQ ID NO:293 is the determined cDNA sequence of 20337, showing homology to Human hepatocyte growth factor activator inhibitor type 2

SEQ ID NO:294 is the determined cDNA sequence of 20338, showing homology to Human cell adhesion molecule CD44

SEQ ID NO:295 is the determined cDNA sequence of 20340, showing homology to Human nuclear factor (erythroid-derived)-like 1

SEQ ID NO:296 is the determined cDNA sequence of 20938, showing homology to Human vinculin mRNA

SEQ ID NO:297 is the determined cDNA sequence of 20939, showing homology to Human elongation factor EF-1-alpha

SEQ ID NO:298 is the determined cDNA sequence of 20940, showing homology to Human nestin gene

SEQ ID NO:299 is the determined cDNA sequence of 20942, showing homology to Human pancreatic ribonuclease

SEQ ID NO:300 is the determined cDNA sequence of 20943, showing homology to Human transcobalamin I

SEQ ID NO:301 is the determined cDNA sequence of 20944, showing homology to Human beta-tubulin

SEQ ID NO:302 is the determined cDNA sequence of 20946, showing homology to Human HS1 protein

SEQ ID NO:303 is the determined cDNA sequence of 20947, showing homology to Human cathepsin B

SEQ ID NO:304 is the determined cDNA sequence of 20948, showing homology to Human testis enhanced gene transcript

SEQ ID NO:305 is the determined cDNA sequence of 20949, showing homology to Human elongation factor EF-1-alpha

SEQ ID NO:306 is the determined cDNA sequence of 20950, showing homology to Human ADP-ribosylation factor 3

SEQ ID NO:307 is the determined cDNA sequence of 20951, showing homology to Human IFP53 or WRS for tryptophanyl-tRNA synthetase

SEQ ID NO:308 is the determined cDNA sequence of 20952, showing homology to Human cyclin-dependent protein kinase

SEQ ID NO:309 is the determined cDNA sequence of 20957, showing homology to Human alpha-tubulin isoform 1

SEQ ID NO:310 is the determined cDNA sequence of 20959, showing homology to Human tyrosine phosphatase-61bp deletion

SEQ ID NO:311 is the determined cDNA sequence of 20966, showing homology to Human tyrosine phosphatase

SEQ ID NO:312 is the determined cDNA sequence of B830P (20976), showing homology to Human nuclear factor NF 45

SEQ ID NO:313 is the determined cDNA sequence of B829P (20977), showing homology to Human delta-6 fatty acid desaturase

SEQ ID NO:314 is the determined cDNA sequence of 20978, showing homology to Human nuclear aconitase

SEQ ID NO:315 is the determined cDNA sequence of clone 23176.

SEQ ID NO:316 is the determined cDNA sequence of clone 23140.

SEQ ID NO:317 is the determined cDNA sequence of clone 23166.

SEQ ID NO:318 is the determined cDNA sequence of clone 23167.
SEQ ID NO:319 is the determined cDNA sequence of clone 23177.
SEQ ID NO:320 is the determined cDNA sequence of clone 23217.
SEQ ID NO:321 is the determined cDNA sequence of clone 23169.
SEQ ID NO:322 is the determined cDNA sequence of clone 23160.
SEQ ID NO:323 is the determined cDNA sequence of clone 23182.
SEQ ID NO:324 is the determined cDNA sequence of clone 23232.
SEQ ID NO:325 is the determined cDNA sequence of clone 23203.
SEQ ID NO:326 is the determined cDNA sequence of clone 23198.
SEQ ID NO:327 is the determined cDNA sequence of clone 23224.
SEQ ID NO:328 is the determined cDNA sequence of clone 23142.
SEQ ID NO:329 is the determined cDNA sequence of clone 23138.
SEQ ID NO:330 is the determined cDNA sequence of clone 23147.
SEQ ID NO:331 is the determined cDNA sequence of clone 23148.
SEQ ID NO:332 is the determined cDNA sequence of clone 23149.
SEQ ID NO:333 is the determined cDNA sequence of clone 23172.
SEQ ID NO:334 is the determined cDNA sequence of clone 23158.
SEQ ID NO:335 is the determined cDNA sequence of clone 23156.
SEQ ID NO:336 is the determined cDNA sequence of clone 23221.
SEQ ID NO:337 is the determined cDNA sequence of clone 23223.
SEQ ID NO:338 is the determined cDNA sequence of clone 23155.
SEQ ID NO:339 is the determined cDNA sequence of clone 23225.
SEQ ID NO:340 is the determined cDNA sequence of clone 23226.
SEQ ID NO:341 is the determined cDNA sequence of clone 23228.
SEQ ID NO:342 is the determined cDNA sequence of clone 23229.
SEQ ID NO:343 is the determined cDNA sequence of clone 23231.
SEQ ID NO:344 is the determined cDNA sequence of clone 23154.
SEQ ID NO:345 is the determined cDNA sequence of clone 23157.
SEQ ID NO:346 is the determined cDNA sequence of clone 23153.

SEQ ID NO:347 is the determined cDNA sequence of clone 23159.
SEQ ID NO:348 is the determined cDNA sequence of clone 23152.
SEQ ID NO:349 is the determined cDNA sequence of clone 23161.
SEQ ID NO:350 is the determined cDNA sequence of clone 23162.
SEQ ID NO:351 is the determined cDNA sequence of clone 23163.
SEQ ID NO:352 is the determined cDNA sequence of clone 23164.
SEQ ID NO:353 is the determined cDNA sequence of clone 23165.
SEQ ID NO:354 is the determined cDNA sequence of clone 23151.
SEQ ID NO:355 is the determined cDNA sequence of clone 23150.
SEQ ID NO:356 is the determined cDNA sequence of clone 23168.
SEQ ID NO:357 is the determined cDNA sequence of clone 23146.
SEQ ID NO:358 is the determined cDNA sequence of clone 23170.
SEQ ID NO:359 is the determined cDNA sequence of clone 23171.
SEQ ID NO:360 is the determined cDNA sequence of clone 23145.
SEQ ID NO:361 is the determined cDNA sequence of clone 23174.
SEQ ID NO:362 is the determined cDNA sequence of clone 23175.
SEQ ID NO:363 is the determined cDNA sequence of clone 23144.
SEQ ID NO:364 is the determined cDNA sequence of clone 23178.
SEQ ID NO:365 is the determined cDNA sequence of clone 23179.
SEQ ID NO:366 is the determined cDNA sequence of clone 23180.
SEQ ID NO:367 is the determined cDNA sequence of clone 23181.
SEQ ID NO:368 is the determined cDNA sequence of clone 23143
SEQ ID NO:369 is the determined cDNA sequence of clone 23183.
SEQ ID NO:370 is the determined cDNA sequence of clone 23184.
SEQ ID NO:371 is the determined cDNA sequence of clone 23185.
SEQ ID NO:372 is the determined cDNA sequence of clone 23186.
SEQ ID NO:373 is the determined cDNA sequence of clone 23187.
SEQ ID NO:374 is the determined cDNA sequence of clone 23190.
SEQ ID NO:375 is the determined cDNA sequence of clone 23189.

SEQ ID NO:376 is the determined cDNA sequence of clone 23202.
SEQ ID NO:378 is the determined cDNA sequence of clone 23191.
SEQ ID NO:379 is the determined cDNA sequence of clone 23188.
SEQ ID NO:380 is the determined cDNA sequence of clone 23194.
SEQ ID NO:381 is the determined cDNA sequence of clone 23196.
SEQ ID NO:382 is the determined cDNA sequence of clone 23195.
SEQ ID NO:383 is the determined cDNA sequence of clone 23193.
SEQ ID NO:384 is the determined cDNA sequence of clone 23199.
SEQ ID NO:385 is the determined cDNA sequence of clone 23200.
SEQ ID NO:386 is the determined cDNA sequence of clone 23192.
SEQ ID NO:387 is the determined cDNA sequence of clone 23201.
SEQ ID NO:388 is the determined cDNA sequence of clone 23141.
SEQ ID NO:389 is the determined cDNA sequence of clone 23139.
SEQ ID NO:390 is the determined cDNA sequence of clone 23204.
SEQ ID NO:391 is the determined cDNA sequence of clone 23205.
SEQ ID NO:392 is the determined cDNA sequence of clone 23206.
SEQ ID NO:393 is the determined cDNA sequence of clone 23207.
SEQ ID NO:394 is the determined cDNA sequence of clone 23208.
SEQ ID NO:395 is the determined cDNA sequence of clone 23209.
SEQ ID NO:396 is the determined cDNA sequence of clone 23210.
SEQ ID NO:397 is the determined cDNA sequence of clone 23211.
SEQ ID NO:398 is the determined cDNA sequence of clone 23212.
SEQ ID NO:399 is the determined cDNA sequence of clone 23214.
SEQ ID NO:400 is the determined cDNA sequence of clone 23215.
SEQ ID NO:401 is the determined cDNA sequence of clone 23216.
SEQ ID NO:402 is the determined cDNA sequence of clone 23137.
SEQ ID NO:403 is the determined cDNA sequence of clone 23218.
SEQ ID NO:404 is the determined cDNA sequence of clone 23220.
SEQ ID NO:405 is the determined cDNA sequence of clone 19462.

SEQ ID NO:406 is the determined cDNA sequence of clone 19430.

SEQ ID NO:407 is the determined cDNA sequence of clone 19407.

SEQ ID NO:408 is the determined cDNA sequence of clone 19448.

SEQ ID NO:409 is the determined cDNA sequence of clone 19447.

SEQ ID NO:410 is the determined cDNA sequence of clone 19426.

SEQ ID NO:411 is the determined cDNA sequence of clone 19441.

SEQ ID NO:412 is the determined cDNA sequence of clone 19454.

SEQ ID NO:413 is the determined cDNA sequence of clone 19463.

SEQ ID NO:414 is the determined cDNA sequence of clone 19419.

SEQ ID NO:415 is the determined cDNA sequence of clone 19434.

SEQ ID NO:416 is the determined extended cDNA sequence of B820P.

SEQ ID NO:417 is the determined extended cDNA sequence of B821P.

SEQ ID NO:418 is the determined extended cDNA sequence of B822P.

SEQ ID NO:419 is the determined extended cDNA sequence of B823P.

SEQ ID NO:420 is the determined extended cDNA sequence of B824P.

SEQ ID NO:421 is the determined extended cDNA sequence of B825P.

SEQ ID NO:422 is the determined extended cDNA sequence of B826P.

SEQ ID NO:423 is the determined extended cDNA sequence of B827P.

SEQ ID NO:424 is the determined extended cDNA sequence of B828P.

SEQ ID NO:425 is the determined extended cDNA sequence of B829P.

SEQ ID NO:426 is the determined extended cDNA sequence of B830P.

SEQ ID NO:427 is the determined cDNA sequence of clone 266B4.

SEQ ID NO:428 is the determined cDNA sequence of clone 22892.

SEQ ID NO:429 is the determined cDNA sequence of clone 266G3.

SEQ ID NO:430 is the determined cDNA sequence of clone 22890.

SEQ ID NO:431 is the determined cDNA sequence of clone 264B4.

SEQ ID NO:432 is the determined cDNA sequence of clone 22883.

SEQ ID NO:433 is the determined cDNA sequence of clone 22882.

SEQ ID NO:434 is the determined cDNA sequence of clone 22880.

SEQ ID NO:435 is the determined cDNA sequence of clone 263G1.
SEQ ID NO:436 is the determined cDNA sequence of clone 263G6.
SEQ ID NO:437 is the determined cDNA sequence of clone 262B2.
SEQ ID NO:438 is the determined cDNA sequence of clone 262B6.
SEQ ID NO:439 is the determined cDNA sequence of clone 22869.
SEQ ID NO:440 is the determined cDNA sequence of clone 21374.
SEQ ID NO:441 is the determined cDNA sequence of clone 21362.
SEQ ID NO:442 is the determined cDNA sequence of clone 21349.
SEQ ID NO:443 is the determined cDNA sequence of clone 21309.
SEQ ID NO:444 is the determined cDNA sequence of clone 21097.
SEQ ID NO:445 is the determined cDNA sequence of clone 21096.
SEQ ID NO:446 is the determined cDNA sequence of clone 21094.
SEQ ID NO:447 is the determined cDNA sequence of clone 21093.
SEQ ID NO:448 is the determined cDNA sequence of clone 21091.
SEQ ID NO:449 is the determined cDNA sequence of clone 21089.
SEQ ID NO:450 is the determined cDNA sequence of clone 21087.
SEQ ID NO:451 is the determined cDNA sequence of clone 21085.
SEQ ID NO:452 is the determined cDNA sequence of clone 21084.
SEQ ID NO:453 is a first partial cDNA sequence of clone 2BT1-40.
SEQ ID NO:454 is a second partial cDNA sequence of clone 2BT1-40.
SEQ ID NO:455 is the determined cDNA sequence of clone 21063.
SEQ ID NO:456 is the determined cDNA sequence of clone 21062.
SEQ ID NO:457 is the determined cDNA sequence of clone 21060.
SEQ ID NO:458 is the determined cDNA sequence of clone 21053.
SEQ ID NO:459 is the determined cDNA sequence of clone 21050.
SEQ ID NO:460 is the determined cDNA sequence of clone 21036.
SEQ ID NO:461 is the determined cDNA sequence of clone 21037.
SEQ ID NO:462 is the determined cDNA sequence of clone 21048.

SEQ ID NO:463 is a consensus DNA sequence of B726P (referred to as B726P-spliced_seq_B726P).

SEQ ID NO:464 is the determined cDNA sequence of a second splice form of B726P (referred to as 27490.seq_B726P).

SEQ ID NO:465 is the determined cDNA sequence of a third splice form of B726P (referred to as 27068.seq_B726P).

SEQ ID NO:466 is the determined cDNA sequence of a second splice form of B726P (referred to as 23113.seq_B726P).

SEQ ID NO:467 is the determined cDNA sequence of a second splice form of B726P (referred to as 23103.seq_B726P).

SEQ ID NO:468 is the determined cDNA sequence of a second splice form of B726P (referred to as 19310.seq_B726P).

SEQ ID NO:469 is the predicted amino acid sequence encoded by the upstream ORF of SEQ ID NO:463.

SEQ ID NO:470 is the predicted amino acid sequence encoded by SEQ ID NO:464.

SEQ ID NO:471 is the predicted amino acid sequence encoded by SEQ ID NO:465.

SEQ ID NO:472 is the predicted amino acid sequence encoded by SEQ ID NO:466.

SEQ ID NO:473 is the predicted amino acid sequence encoded by SEQ ID NO:467.

SEQ ID NO:474 is the determined cDNA sequence for an alternative splice form of B726P.

SEQ ID NO:475 is the amino acid sequence encoded by SEQ ID NO:474.

SEQ ID NO:476 is the isolated cDNA sequence of B720P.

SEQ ID NO:477 is the cDNA sequence of a known keratin gene.

SEQ ID NO:478 is the amino acid sequence encoded by SEQ ID NO:477.

SEQ ID NO:479 is the determined cDNA sequence for clone 19465.

SEQ ID NO:480 and 481 are PCR primers.

SEQ ID NO:482 is the cDNA sequence for the expressed downstream ORF of B726P.

SEQ ID NO:483 is the amino acid sequence for the expressed recombinant downstream ORF of B726P.

SEQ ID NO:484 is the determined full-length cDNA sequence for B720P.

SEQ ID NO:485 is the amino acid sequence encoded by SEQ ID NO:484.

SEQ ID NO:486 is the determined cDNA sequence of a truncated form of B720P, referred to as B720P-tr.

SEQ ID NO:487 is the amino acid sequence of B720P-tr.

SEQ ID NO:488 is the amino acid sequence of a naturally processed epitope of B726P recognized by B726P-specific CTL.

SEQ ID NO:489 is a DNA sequence encoding the B726P epitope set forth in SEQ ID NO:488.

SEQ ID NO:490 is a DNA sequence encoding a fusion protein wherein mammaglobin is fused to the B726P combined upstream and downstream open reading frame (ORF) (the amino acid sequence of the B726P combined ORF is disclosed herein as SEQ ID NO:475 which is encoded by the DNA sequence of SEQ ID NO:474).

SEQ ID NO:491 is a DNA sequence encoding a fusion protein wherein mammaglobin is fused to the B726P upstream ORF (the amino acid sequence of the B726P upstream ORF is disclosed herein as SEQ ID NO:469 which is encoded by the DNA sequence of SEQ ID NO:463).

SEQ ID NO:492 is a DNA sequence encoding a fusion protein wherein mammaglobin is fused to the B726P downstream ORF (the amino acid sequence of the B726P downstream ORF is disclosed herein as SEQ ID NO:176 which is encoded by the DNA sequence of SEQ ID NO:175).

SEQ ID NO:493 is the amino acid sequence encoded by the DNA sequence of SEQ ID NO:490.

SEQ ID NO:494 is the amino acid sequence encoded by the DNA sequence of SEQ ID NO:491.

SEQ ID NO:495 is the amino acid sequence encoded by the DNA sequence of SEQ ID NO:492.

SEQ ID NO:496 is amino acids 59-78 of SEQ ID NO:493.

SEQ ID NO:497 is amino acids 55-69 of SEQ ID NO:493.

SEQ ID NO:498 is amino acids 13-33 of SEQ ID NO:493.

SEQ ID NO:499 is amino acids 41-60 of SEQ ID NO:493.

SEQ ID NO:500 is amino acids 2-10 of SEQ ID NO:493.

SEQ ID NO:501 is amino acids 47-59 of SEQ ID NO:493.

SEQ ID NO:502 is amino acids 62-74 of SEQ ID NO:493.

SEQ ID NO:503 is amino acids 1-93 of SEQ ID NO:493.

SEQ ID NO:504 is the full-length cDNA sequence for B718P.

SEQ ID NO:505 is the cDNA sequence of the open reading frame of B718P including stop codon.

SEQ ID NO:506 is the cDNA sequence of the open reading frame of B718P without stop codon.

SEQ ID NO:507 is the full-length amino acid sequence of B718P.

SEQ ID NO:508 represents amino acids 1-158 of SEQ ID NO:507.

SEQ ID NO:509 represents amino acids 159-243 of SEQ ID NO:509.

SEQ ID NO:510 is the entire cDNA sequence of the open reading frame, including stop codon, of a first variant of B723P, referred to as B723P-short.

SEQ ID NO:511 is the entire cDNA sequence of the open reading frame, without stop codon, of a first variant of B723P, referred to as B723P-short.

SEQ ID NO:512 is the entire cDNA sequence of the open reading frame, including stop codon, of a second variant of B723P, referred to as B723P-long.

SEQ ID NO:513 is the entire cDNA sequence of the open reading frame, without stop codon, of a second variant of B723P, referred to as B723P-long.

SEQ ID NO:514 is the amino acid sequence of B723P-short.

SEQ ID NO:515 is the amino acid sequence of B723P-long.

SEQ ID NO:516 is amino acids 1-197 of B723P-short.

SEQ ID NO:517 is amino acids 1-232 of B723P-long.

SEQ ID NO:518 is amino acids 198-243 of B723P-short.

SEQ ID NO:519 is amino acids 218-243 of B723P-short.

SEQ ID NO:520 – 533 are the DNA sequences of epitopes of B726P.

SEQ ID NO:534-547 are the amino acid sequences of epitopes of B726P.

SEQ ID NO:548 is the cDNA sequence of B726P Combined ORF coding_ region for expression in *E. coli*.

SEQ ID NO:549 is the cDNA sequence of B726P Upstream ORF coding_ region for expression in *E. coli*.

SEQ ID NO:550 is the cDNA sequence of B726P Downstream ORF coding_ region for expression in *E. coli*.

SEQ ID NO:551 is the amino acid sequence of B726P Downstream ORF encoded by the cDNA set forth in SEQ ID NO:550.

SEQ ID NO:552 is the amino acid sequence of B726P Upstream ORF with HIS, encoded by the cDNA set forth in SEQ ID NO:549.

SEQ ID NO:553 is the amino acid sequence of B726P Combined ORF correct, encoded by the cDNA set forth in SEQ ID NO:548.

SEQ ID NO:554-563 are PCR primers as described in Example 8.

SEQ ID NO:564 is the cDNA sequence for NY-BR-1, an extended sequence of B726P.

SEQ ID NO:565 is the amino acid sequence for NY-BR-1, an extended sequence of B726P, and encoded by the nucleotide sequence set forth in SEQ ID NO:564.

SEQ ID NO:566 is the cDNA sequence for B726P XC coding region with changes.

SEQ ID NO:567 is the cDNA sequence for B726P XB clone 83686 with 2 changes from the published NY-BR-1 sequence in SEQ ID NO:564.

SEQ ID NO:568 is the cDNA sequence for B726P XB clone 84330 with 4 changes from the published NY-BR-1 sequence in SEQ ID NO:564.

SEQ ID NO:569 is the cDNA sequence for B726P XB clone 84328 with 3 changes from the published NY-BR-1 sequence in SEQ ID NO:564.

SEQ ID NO:570 is the amino acid sequence for B726P XB clone 84328, encoded by the sequence set forth in SEQ ID NO:569.

SEQ ID NO:571 is the amino acid sequence for B726P XB clone 84330, encoded by the sequence set forth in SEQ ID NO:568.

SEQ ID NO:572 is the amino acid sequence for B726P XB clone 83686, encoded by the sequence set forth in SEQ ID NO:567.

SEQ ID NO:573 is the amino acid sequence for B726P XC, encoded by the sequence set forth in SEQ ID NO:566.

SEQ ID NO:574-575 are PCR primers as described in Example 12.

SEQ ID NO:576 is the full-length cDNA sequence for NY-BR-1.1.

SEQ ID NO:577 is the full-length amino acid sequence for NY-BR-1.1, encoded by the nucleotide sequence set forth in SEQ ID NO:576.

SEQ ID NO:578 is amino acids 289-308 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A2.1 antibody.

SEQ ID NO:579 is amino acids 225-244 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A19.1 antibody.

SEQ ID NO:580 is amino acids 232-252 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A19.1 and the 220A43 antibodies.

SEQ ID NO:581 is amino acids 73-92 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A94.1 antibody.

SEQ ID NO:582 is amino acids 145-164 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A151.1 and 220A86 antibodies.

SEQ ID NO:583 is amino acids 153-172 of the B726P downstream ORF and corresponds to the peptide recognized by the 220A151.1 and 220A86 antibodies.

SEQ ID NO:584 is amino acids 1-20 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:585 is amino acids 9-28 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:586 is amino acids 17-36 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:587 is amino acids 24-44 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:588 is amino acids 97-116 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:589 is amino acids 105-124 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:590 is amino acids 113-132 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:591 is amino acids 121-140 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:592 is amino acids 129-148 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:593 is amino acids 137-156 of the B726P downstream ORF and corresponds to the peptide recognized by purified B726 polyclonal antibodies.

SEQ ID NO:594 is the amino acid sequence of peptide #2732 and corresponds to amino acids 1-20 of the B726P downstream ORF.

SEQ ID NO:595 is the amino acid sequence of peptide #2733 and corresponds to amino acids 11-30 of the B726P downstream ORF.

SEQ ID NO:596 is the amino acid sequence of peptide #2734 and corresponds to amino acids 21-40 of the B726P downstream ORF.

SEQ ID NO:597 is the amino acid sequence of peptide #2735 and corresponds to amino acids 31-50 of the B726P downstream ORF.

SEQ ID NO:598 is the amino acid sequence of peptide #2736 and corresponds to amino acids 41-60 of the B726P downstream ORF.

SEQ ID NO:599 is the amino acid sequence of peptide #2737 and corresponds to amino acids 51-70 of the B726P downstream ORF.

SEQ ID NO:600 is the amino acid sequence of peptide #2738 and corresponds to amino acids 61-80 of the B726P downstream ORF.

SEQ ID NO:601 is the amino acid sequence of peptide #2739 and corresponds to amino acids 71-90 of the B726P downstream ORF.

SEQ ID NO:602 is the amino acid sequence of peptide #2740 and corresponds to amino acids 81-100 of the B726P downstream ORF.

SEQ ID NO:603 is the amino acid sequence of peptide #2741 and corresponds to amino acids 91-110 of the B726P downstream ORF.

SEQ ID NO:604 is the amino acid sequence of peptide #2742 and corresponds to amino acids 101-120 of the B726P downstream ORF.

SEQ ID NO:605 is the amino acid sequence of peptide #2743 and corresponds to amino acids 111-130 of the B726P downstream ORF.

SEQ ID NO:606 is the amino acid sequence of peptide #2744 and corresponds to amino acids 121-140 of the B726P downstream ORF.

SEQ ID NO:607 is the amino acid sequence of peptide #2745 and corresponds to amino acids 130-151 of the B726P downstream ORF.

SEQ ID NO:608 is the amino acid sequence of peptide #2746 and corresponds to amino acids 141-160 of the B726P downstream ORF.

SEQ ID NO:609 is the amino acid sequence of peptide #2747 and corresponds to amino acids 151-170 of the B726P downstream ORF.

SEQ ID NO:610 is the amino acid sequence of peptide #2748 and corresponds to amino acids 161-180 of the B726P downstream ORF.

SEQ ID NO:611 is the amino acid sequence of peptide #2749 and corresponds to amino acids 170-190 of the B726P downstream ORF.

SEQ ID NO:612 is the amino acid sequence of peptide #2750 and corresponds to amino acids 181-200 of the B726P downstream ORF.

SEQ ID NO:613 is the amino acid sequence of peptide #2751 and corresponds to amino acids 191-210 of the B726P downstream ORF.

SEQ ID NO:614 is the amino acid sequence of peptide #2752 and corresponds to amino acids 201-220 of the B726P downstream ORF.

SEQ ID NO:615 is the amino acid sequence of peptide #2753 and corresponds to amino acids 211-230 of the B726P downstream ORF.

SEQ ID NO:616 is the amino acid sequence of peptide #2765 and corresponds to amino acids 221-240 of the B726P downstream ORF.

SEQ ID NO:617 is the amino acid sequence of peptide #2766 and corresponds to amino acids 231-250 of the B726P downstream ORF.

SEQ ID NO:618 is the amino acid sequence of peptide #2767 and corresponds to amino acids 240-260 of the B726P downstream ORF.

SEQ ID NO:619 is the amino acid sequence of peptide #2768 and corresponds to amino acids 251-270 of the B726P downstream ORF.

SEQ ID NO:620 is the amino acid sequence of peptide #2769 and corresponds to amino acids 261-280 of the B726P downstream ORF.

SEQ ID NO:621 is the amino acid sequence of peptide #2770 and corresponds to amino acids 271-290 of the B726P downstream ORF.

SEQ ID NO:622 is the amino acid sequence of peptide #2771 and corresponds to amino acids 281-300 of the B726P downstream ORF.

SEQ ID NO:623 is the amino acid sequence of peptide #2772 and corresponds to amino acids 291-310 of the B726P downstream ORF.

SEQ ID NO:624 is the amino acid sequence of peptide #2773 and corresponds to amino acids 298-317 of the B726P downstream ORF.

SEQ ID NO:625 is the amino acid sequence of peptide #3535 of B726P.

SEQ ID NO:626 is the amino acid sequence of peptide #3536 of B726P.

SEQ ID NO:627 is the amino acid sequence of peptide #3534 of B726P.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed generally to compositions and their use in the therapy and diagnosis of cancer, particularly breast cancer. As described further below, illustrative compositions of the present invention include, but are not restricted to, polypeptides, particularly immunogenic polypeptides, polynucleotides encoding such polypeptides, antibodies and other binding agents, antigen presenting cells (APCs) and immune system cells (*e.g.*, T cells).

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, et al. Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Maniatis et al. Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Perbal, A Practical Guide to Molecular Cloning (1984).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

Polypeptide Compositions

As used herein, the term "polypeptide" " is used in its conventional meaning, *i.e.*, as a sequence of amino acids. The polypeptides are not limited to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide, and such terms may be used interchangeably herein unless specifically

indicated otherwise. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. A polypeptide may be an entire protein, or a subsequence thereof. Particular polypeptides of interest in the context of this invention are amino acid subsequences comprising epitopes, *i*.e., antigenic determinants substantially responsible for the immunogenic properties of a polypeptide and being capable of evoking an immune response.

Particularly illustrative polypeptides of the present invention comprise those encoded by a polynucleotide sequence set forth in any one of SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576, or a sequence that hybridizes under moderately stringent conditions, or, alternatively, under highly stringent conditions, to a polynucleotide sequence set forth in any one of SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576. Certain other illustrative polypeptides of the invention comprise amino acid sequences as set forth in any one of SEQ ID NO: 62, 176, 179, 181, 469-473, 475, 478, 483, 485, 487, 488, 493-503, 507-509, 514-519, 534-547, 551-553, 565, 570-573, and 577-627.

The polypeptides of the present invention are sometimes herein referred to as breast tumor proteins or breast tumor polypeptides, as an indication that their identification has been based at least in part upon their increased levels of expression in breast tumor samples. Thus, a "breast tumor polypeptide" or "breast tumor protein," refers generally to a polypeptide sequence of the present invention, or a polynucleotide sequence encoding such a polypeptide, that is expressed in a substantial proportion of breast tumor samples, for example preferably greater than about 20%, more preferably greater than about 30%, and most preferably greater than about 50% or more of breast tumor samples tested, at a level that is at least two fold, and preferably at least five fold, greater than the level of expression in normal tissues, as determined using a representative assay provided herein. A

breast tumor polypeptide sequence of the invention, based upon its increased level of expression in tumor cells, has particular utility both as a diagnostic marker as well as a therapeutic target, as further described below.

In certain preferred embodiments, the polypeptides of the invention are immunogenic, *i.e.*, they react detectably within an immunoassay (such as an ELISA or T-cell stimulation assay) with antisera and/or T-cells from a patient with breast cancer. Screening for immunogenic activity can be performed using techniques well known to the skilled artisan. For example, such screens can be performed using methods such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In one illustrative example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example, ^{125}I-labeled Protein A.

As would be recognized by the skilled artisan, immunogenic portions of the polypeptides disclosed herein are also encompassed by the present invention. An "immunogenic portion," as used herein, is a fragment of an immunogenic polypeptide of the invention that itself is immunologically reactive (*i.e.*, specifically binds) with the B-cells and/or T-cell surface antigen receptors that recognize the polypeptide. Immunogenic portions may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. As used herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.*, they react with the protein in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well-known techniques.

In one preferred embodiment, an immunogenic portion of a polypeptide of the present invention is a portion that reacts with antisera and/or T-cells at a level that is not substantially less than the reactivity of the full-length polypeptide (*e.g.*, in an ELISA and/or

T-cell reactivity assay). Preferably, the level of immunogenic activity of the immunogenic portion is at least about 50%, preferably at least about 70% and most preferably greater than about 90% of the immunogenicity for the full-length polypeptide. In some instances, preferred immunogenic portions will be identified that have a level of immunogenic activity greater than that of the corresponding full-length polypeptide, *e.g.*, having greater than about 100% or 150% or more immunogenic activity.

In certain other embodiments, illustrative immunogenic portions may include peptides in which an N-terminal leader sequence and/or transmembrane domain have been deleted. Other illustrative immunogenic portions will contain a small N- and/or C-terminal deletion (*e.g.*, 1-30 amino acids, preferably 5-15 amino acids), relative to the mature protein.

In another embodiment, a polypeptide composition of the invention may also comprise one or more polypeptides that are immunologically reactive with T cells and/or antibodies generated against a polypeptide of the invention, particularly a polypeptide having an amino acid sequence disclosed herein, or to an immunogenic fragment or variant thereof.

In another embodiment of the invention, polypeptides are provided that comprise one or more polypeptides that are capable of eliciting T cells and/or antibodies that are immunologically reactive with one or more polypeptides described herein, or one or more polypeptides encoded by contiguous nucleic acid sequences contained in the polynucleotide sequences disclosed herein, or immunogenic fragments or variants thereof, or to one or more nucleic acid sequences which hybridize to one or more of these sequences under conditions of moderate to high stringency.

The present invention, in another aspect, provides polypeptide fragments comprising at least about 5, 10, 15, 20, 25, 50, or 100 contiguous amino acids, or more, including all intermediate lengths, of a polypeptide compositions set forth herein, such as those set forth in SEQ ID NO: 62, 176, 179, 181, 469-473, 475, 478, 483, 485, 487, 488, 493-503, 507-509, 514-519, 534-547, 551-553, 565, 570-573, and 577-627, or those encoded by a polynucleotide sequence set forth in a sequence of SEQ ID NO: 1-61, 63-175,

178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576.

In another aspect, the present invention provides variants of the polypeptide compositions described herein. Polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to a polypeptide sequences set forth herein.

In one preferred embodiment, the polypeptide fragments and variants provide by the present invention are immunologically reactive with an antibody and/or T-cell that reacts with a full-length polypeptide specifically set for the herein.

In another preferred embodiment, the polypeptide fragments and variants provided by the present invention exhibit a level of immunogenic activity of at least about 50%, preferably at least about 70%, and most preferably at least about 90% or more of that exhibited by a full-length polypeptide sequence specifically set forth herein.

A polypeptide "variant," as the term is used herein, is a polypeptide that typically differs from a polypeptide specifically disclosed herein in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences of the invention and evaluating their immunogenic activity as described herein and/or using any of a number of techniques well known in the art.

For example, certain illustrative variants of the polypeptides of the invention include those in which one or more portions, such as an N-terminal leader sequence or transmembrane domain, have been removed. Other illustrative variants include variants in which a small portion (*e.g.*, 1-30 amino acids, preferably 5-15 amino acids) has been removed from the N- and/or C-terminal of the mature protein.

In many instances, a variant will contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially

unchanged. As described above, modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics, *e.g.*, with immunogenic characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, immunogenic variant or portion of a polypeptide of the invention, one skilled in the art will typically change one or more of the codons of the encoding DNA sequence according to Table 1.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been

assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their

hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition, any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Amino acid substitutions may further be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

As noted above, polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-

His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

When comparing polypeptide sequences, two sequences are said to be "identical" if the sequence of amino acids in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenes pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* *5*:151-153; Myers, E.W. and Muller W. (1988) *CABIOS 4*:11-17; Robinson, E.D. (1971) *Comb. Theor 11*:105; Santou, N. Nes, M. (1987) *Mol. Biol. Evol. 4*:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad., Sci. USA 80*:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP,

BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nucl. Acids Res.* 25:3389-3402 and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and polypeptides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. For amino acid sequences, a scoring matrix can be used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment.

In one preferred approach, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Within other illustrative embodiments, a polypeptide may be a fusion polypeptide that comprises multiple polypeptides as described herein, or that comprises at

least one polypeptide as described herein and an unrelated sequence, such as a known tumor protein. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the polypeptide or to enable the polypeptide to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

Fusion polypeptides may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion polypeptide is expressed as a recombinant polypeptide, allowing the production of increased levels, relative to a non-fused polypeptide, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid

sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

The fusion polypeptide can comprise a polypeptide as described herein together with an unrelated immunogenic protein, such as an immunogenic protein capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see*, for example, Stoute et al. *New Engl. J. Med., 336*:86-91, 1997).

In one preferred embodiment, the immunological fusion partner is derived from a Mycobacterium sp., such as a Mycobacterium tuberculosis-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences is described in U.S. Patent Application 60/158,585, the disclosure of which is incorporated herein by reference in its entirety. Briefly, Ra12 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been described (for example, U.S. Patent Application 60/158,585; see also, Skeiky *et al.*, *Infection and Immun.* (1999) 67:3998-4007, incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as a soluble polypeptides throughout the purification process. Moreover, Ra12 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One

preferred Ral2 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other preferred Ral2 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ral2 polypeptide. Ral2 polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a Ral2 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ral2 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ral2 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ral2 polypeptide or a portion thereof.

Within other preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium Haemophilus influenza B (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.,* the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemaglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene 43*:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-

terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology 10*:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

Yet another illustrative embodiment involves fusion polypeptides, and the polynucleotides encoding them, wherein the fusion partner comprises a targeting signal capable of directing a polypeptide to the endosomal/lysosomal compartment, as described in U.S. Patent No. 5,633,234. An immunogenic polypeptide of the invention, when fused with this targeting signal, will associate more efficiently with MHC class II molecules and thereby provide enhanced in vivo stimulation of $CD4^+$ T-cells specific for the polypeptide.

Polypeptides of the invention are prepared using any of a variety of well known synthetic and/or recombinant techniques, the latter of which are further described below. Polypeptides, portions and other variants generally less than about 150 amino acids can be generated by synthetic means, using techniques well known to those of ordinary skill in the art. In one illustrative example, such polypeptides are synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc. 85*:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptide compositions (including fusion polypeptides) of the invention are isolated. An "isolated" polypeptide is one that is removed from its original environment. For example, a naturally-occurring protein or polypeptide is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably,

such polypeptides are also purified, *e.g.*, are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

Polynucleotide Compositions

The present invention, in other aspects, provides polynucleotide compositions. The terms "DNA" and "polynucleotide" are used essentially interchangeably herein to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. "Isolated," as used herein, means that a polynucleotide is substantially away from other coding sequences, and that the DNA molecule does not contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

As will be understood by those skilled in the art, the polynucleotide compositions of this invention can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the hand of man.

As will be also recognized by the skilled artisan, polynucleotides of the invention may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a polypeptide/protein of the invention or a portion thereof) or may

comprise a sequence that encodes a variant or derivative, preferably and immunogenic variant or derivative, of such a sequence.

Therefore, according to another aspect of the present invention, polynucleotide compositions are provided that comprise some or all of a polynucleotide sequence set forth in any one of SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576, complements of a polynucleotide sequence set forth in any one of SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576, and degenerate variants of a polynucleotide sequence set forth in any one of SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576. In certain preferred embodiments, the polynucleotide sequences set forth herein encode immunogenic polypeptides, as described above.

In other related embodiments, the present invention provides polynucleotide variants having substantial identity to the sequences disclosed herein in SEQ ID NO: 1-61, 63-175, 178, 180, 182-468, 474, 476, 477, 479, 482, 484, 486, 489-492, 504-506, 510-513, 520-533, 548-550, 564, 566-569, and 576, for example those comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a polynucleotide sequence of this invention using the methods described herein, (*e.g.*, BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the immunogenicity of the polypeptide encoded by the variant polynucleotide is not substantially diminished relative to a polypeptide encoded by a polynucleotide sequence specifically set forth herein). The

term "variants" should also be understood to encompasses homologous genes of xenogenic origin.

In additional embodiments, the present invention provides polynucleotide fragments comprising various lengths of contiguous stretches of sequence identical to or complementary to one or more of the sequences disclosed herein. For example, polynucleotides are provided by this invention that comprise at least about 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500 or 1000 or more contiguous nucleotides of one or more of the sequences disclosed herein as well as all intermediate lengths there between. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 16, 17, 18, 19, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through 200-500; 500-1,000, and the like.

In another embodiment of the invention, polynucleotide compositions are provided that are capable of hybridizing under moderate to high stringency conditions to a polynucleotide sequence provided herein, or a fragment thereof, or a complementary sequence thereof. Hybridization techniques are well known in the art of molecular biology. For purposes of illustration, suitable moderately stringent conditions for testing the hybridization of a polynucleotide of this invention with other polynucleotides include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-60°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. One skilled in the art will understand that the stringency of hybridization can be readily manipulated, such as by altering the salt content of the hybridization solution and/or the temperature at which the hybridization is performed. For example, in another embodiment, suitable highly stringent hybridization conditions include those described above, with the exception that the temperature of hybridization is increased, *e.g.*, to 60-65°C or 65-70°C.

In certain preferred embodiments, the polynucleotides described above, *e.g.*, polynucleotide variants, fragments and hybridizing sequences, encode polypeptides that are immunologically cross-reactive with a polypeptide sequence specifically set forth herein.

In other preferred embodiments, such polynucleotides encode polypeptides that have a level of immunogenic activity of at least about 50%, preferably at least about 70%, and more preferably at least about 90% of that for a polypeptide sequence specifically set forth herein.

The polynucleotides of the present invention, or fragments thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative polynucleotide segments with total lengths of about 10,000, about 5000, about 3000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this invention.

When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research

Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenes pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* 5:151-153; Myers, E.W. and Muller W. (1988) *CABIOS* 4:11-17; Robinson, E.D. (1971) *Comb. Theor* 11:105; Santou, N. Nes, M. (1987) *Mol. Biol. Evol.* 4:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad., Sci. USA 80*:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nucl. Acids Res.* 25:3389-3402 and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the

sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, is employed for the preparation of immunogenic variants and/or derivatives of the polypeptides described herein. By this approach, specific

modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provides a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter one or more properties of the encoded polypeptide, such as the immunogenicity of a polypeptide vaccine. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector that includes within its sequence a DNA sequence that encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson,

1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety.

In another approach for the production of polypeptide variants of the present invention, recursive sequence recombination, as described in U.S. Patent No. 5,837,458, may be employed. In this approach, iterative cycles of recombination and screening or selection are performed to "evolve" individual polynucleotide variants of the invention having, for example, enhanced immunogenic activity.

In other embodiments of the present invention, the polynucleotide sequences provided herein can be advantageously used as probes or primers for nucleic acid hybridization. As such, it is contemplated that nucleic acid segments that comprise a sequence region of at least about 15 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 15 nucleotide long contiguous sequence disclosed herein will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000 (including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to a sequence of interest will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are also envisioned, such as the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Polynucleotide molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so (including intermediate lengths as well), identical or complementary to a polynucleotide sequence disclosed herein, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. This would allow a gene product, or fragment

thereof, to be analyzed, both in diverse cell types and also in various bacterial cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 15 and about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 15-25 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 15 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 25 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequences set forth herein, or to any continuous portion of the sequences, from about 15-25 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors. For example, one may wish to employ primers from towards the termini of the total sequence.

Small polynucleotide segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patent 4,683,202 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

The nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of the entire gene or gene

fragments of interest. Depending on the application envisioned, one will typically desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by a salt concentration of from about 0.02 M to about 0.15 M salt at temperatures of from about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating related sequences.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent (reduced stringency) hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ salt conditions such as those of from about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

According to another embodiment of the present invention, polynucleotide compositions comprising antisense oligonucleotides are provided. Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, provide a therapeutic approach by which a disease can be treated by inhibiting the synthesis of proteins that contribute to the disease. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S.

Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski *et al.*, Science. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, Brain Res Mol Brain Res. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Therefore, in certain embodiments, the present invention provides oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to polynucleotide sequence described herein, or a complement thereof. In one embodiment, the antisense oligonucleotides comprise DNA or derivatives thereof. In another embodiment, the oligonucleotides comprise RNA or derivatives thereof. In a third embodiment, the oligonucleotides are modified DNAs comprising a phosphorothioated modified backbone. In a fourth embodiment, the oligonucleotide sequences comprise peptide nucleic acids or derivatives thereof. In each case, preferred compositions comprise a sequence region that is complementary, and more preferably substantially-complementary, and even more preferably, completely complementary to one or more portions of polynucleotides disclosed herein. Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense compositions may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those which are at or near the AUG translation initiation codon, and those sequences which are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer

analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, Nucleic Acids Res. 1997, 25(17):3389-402).

The use of an antisense delivery method employing a short peptide vector, termed MPG (27 residues), is also contemplated. The MPG peptide contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain from the nuclear localization sequence of SV40 T-antigen (Morris *et al.*, Nucleic Acids Res. 1997 Jul 15;25(14):2730-6). It has been demonstrated that several molecules of the MPG peptide coat the antisense oligonucleotides and can be delivered into cultured mammalian cells in less than 1 hour with relatively high efficiency (90%). Further, the interaction with MPG strongly increases both the stability of the oligonucleotide to nuclease and the ability to cross the plasma membrane.

According to another embodiment of the invention, the polynucleotide compositions described herein are used in the design and preparation of ribozyme molecules for inhibiting expression of the tumor polypeptides and proteins of the present invention in tumor cells. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J Mol Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through

the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7305-9). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.*, Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis δ virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec

1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, Proc Natl Acad Sci U S A. 1991 Oct 1;88(19):8826-30; Collins and Olive, Biochemistry. 1993 Mar 23;32(11):2795-9); and an example of the Group I intron is described in (U. S. Patent 4,987,071). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into

other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells Ribozymes expressed from such promoters have been shown to function in mammalian cells. Such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

In another embodiment of the invention, peptide nucleic acids (PNAs) compositions are provided. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, Antisense Nucleic Acid Drug Dev. 1997 7(4) 431-37). PNA is able to be utilized in a number methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding

RNA or DNA sequences and have utilities that are not inherent to RNA or DNA. A review of PNA including methods of making, characteristics of, and methods of using, is provided by Corey (*Trends Biotechnol* 1997 Jun;15(6):224-9). As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of the ACE mRNA sequence, and such PNA compositions may be used to regulate, alter, decrease, or reduce the translation of ACE-specific mRNA, and thereby alter the level of ACE activity in a host cell to which such PNA compositions have been administered.

PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen *et al.*, *Science* 1991 Dec 6;254(5037):1497-500; Hanvey *et al.*, Science. 1992 Nov 27;258(5087):1481-5; Hyrup and Nielsen, Bioorg Med Chem. 1996 Jan;4(1):5-23). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc or Fmoc protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used.

PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, MA). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton *et al.*, Bioorg Med Chem. 1995 Apr;3(4):437-45). The manual protocol lends itself to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs.

As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, the presence of adjacent purines can lead to deletions of one or more residues in the product. In expectation of this difficulty, it is suggested that, in producing PNAs with adjacent purines, one should repeat the coupling of residues likely to be added inefficiently. This should be followed by the purification of

PNAs by reverse-phase high-pressure liquid chromatography, providing yields and purity of product similar to those observed during the synthesis of peptides.

Modifications of PNAs for a given application may be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic acid group to the exposed N-terminal amine. Alternatively, PNAs can be modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry. Several studies have made and utilized modifications of PNAs (for example, Norton *et al.*, Bioorg Med Chem. 1995 Apr;3(4):437-45; Petersen *et al.*, J Pept Sci. 1995 May-Jun;1(3):175-83; Orum *et al.*, Biotechniques. 1995 Sep;19(3):472-80; Footer *et al.*, Biochemistry. 1996 Aug 20;35(33):10673-9; Griffith *et al.*, Nucleic Acids Res. 1995 Aug 11;23(15):3003-8; Pardridge *et al.*, Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5592-6; Boffa *et al.*, Proc Natl Acad Sci U S A. 1995 Mar 14;92(6):1901-5; Gambacorti-Passerini *et al.*, Blood. 1996 Aug 15;88(4):1411-7; Armitage *et al.*, Proc Natl Acad Sci U S A. 1997 Nov 11;94(23):12320-5; Seeger *et al.*, Biotechniques. 1997 Sep;23(3):512-7). U.S. Patent No. 5,700,922 discusses PNA-DNA-PNA chimeric molecules and their uses in diagnostics, modulating protein in organisms, and treatment of conditions susceptible to therapeutics.

Methods of characterizing the antisense binding properties of PNAs are discussed in Rose (Anal Chem. 1993 Dec 15;65(24):3545-9) and Jensen *et al.* (Biochemistry. 1997 Apr 22;36(16):5072-7). Rose uses capillary gel electrophoresis to determine binding of PNAs to their complementary oligonucleotide, measuring the relative binding kinetics and stoichiometry. Similar types of measurements were made by Jensen *et al.* using BIAcore™ technology.

Other applications of PNAs that have been described and will be apparent to the skilled artisan include use in DNA strand invasion, antisense inhibition, mutational analysis, enhancers of transcription, nucleic acid purification, isolation of transcriptionally

active genes, blocking of transcription factor binding, genome cleavage, biosensors, *in situ* hybridization, and the like.

Polynucleotide Identification, Characterization and Expression

Polynucleotides compositions of the present invention may be identified, prepared and/or manipulated using any of a variety of well established techniques (see generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989, and other like references). For example, a polynucleotide may be identified, as described in more detail below, by screening a microarray of cDNAs for tumor-associated expression (*i.e.,* expression that is at least two fold greater in a tumor than in normal tissue, as determined using a representative assay provided herein). Such screens may be performed, for example, using the microarray technology of Affymetrix, Inc. (Santa Clara, CA) according to the manufacturer's instructions (and essentially as described by Schena et al., *Proc. Natl. Acad. Sci. USA 93*:10614-10619, 1996 and Heller et al., *Proc. Natl. Acad. Sci. USA 94*:2150-2155, 1997). Alternatively, polynucleotides may be amplified from cDNA prepared from cells expressing the proteins described herein, such as tumor cells.

Many template dependent processes are available to amplify a target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety. Briefly, in PCR™, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase (*e.g.*, *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction product

and the process is repeated. Preferably reverse transcription and PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Any of a number of other template dependent processes, many of which are variations of the PCR ™ amplification technique, are readily known and available in the art. Illustratively, some such methods include the ligase chain reaction (referred to as LCR), described, for example, in Eur. Pat. Appl. Publ. No. 320,308 and U.S. Patent No. 4,883,750; Qbeta Replicase, described in PCT Intl. Pat. Appl. Publ. No. PCT/US87/00880; Strand Displacement Amplification (SDA) and Repair Chain Reaction (RCR). Still other amplification methods are described in Great Britain Pat. Appl. No. 2 202 328, and in PCT Intl. Pat. Appl. Publ. No. PCT/US89/01025. Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (PCT Intl. Pat. Appl. Publ. No. WO 88/10315), including nucleic acid sequence based amplification (NASBA) and 3SR. Eur. Pat. Appl. Publ. No. 329,822 describes a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA). PCT Intl. Pat. Appl. Publ. No. WO 89/06700 describes a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. Other amplification methods such as "RACE" (Frohman, 1990), and "one-sided PCR" (Ohara, 1989) are also well-known to those of skill in the art.

An amplified portion of a polynucleotide of the present invention may be used to isolate a full length gene from a suitable library (*e.g.*, a tumor cDNA library) using well known techniques. Within such techniques, a library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, a library is size-selected to include larger molecules. Random primed libraries may also be preferred for identifying 5' and upstream regions of genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences.

For hybridization techniques, a partial sequence may be labeled (*e.g.*, by nick-translation or end-labeling with ^{32}P) using well known techniques. A bacterial or

bacteriophage library is then generally screened by hybridizing filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (*see* Sambrook et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis. cDNA clones may be analyzed to determine the amount of additional sequence by, for example, PCR using a primer from the partial sequence and a primer from the vector. Restriction maps and partial sequences may be generated to identify one or more overlapping clones. The complete sequence may then be determined using standard techniques, which may involve generating a series of deletion clones. The resulting overlapping sequences can then assembled into a single contiguous sequence. A full length cDNA molecule can be generated by ligating suitable fragments, using well known techniques.

Alternatively, amplification techniques, such as those described above, can be useful for obtaining a full length coding sequence from a partial cDNA sequence. One such amplification technique is inverse PCR (*see* Triglia et al., *Nucl. Acids Res. 16*:8186, 1988), which uses restriction enzymes to generate a fragment in the known region of the gene. The fragment is then circularized by intramolecular ligation and used as a template for PCR with divergent primers derived from the known region. Within an alternative approach, sequences adjacent to a partial sequence may be retrieved by amplification with a primer to a linker sequence and a primer specific to a known region. The amplified sequences are typically subjected to a second round of amplification with the same linker primer and a second primer specific to the known region. A variation on this procedure, which employs two primers that initiate extension in opposite directions from the known sequence, is described in WO 96/38591. Another such technique is known as "rapid amplification of cDNA ends" or RACE. This technique involves the use of an internal primer and an external primer, which hybridizes to a polyA region or vector sequence, to identify sequences that are 5' and 3' of a known sequence. Additional techniques include capture PCR (Lagerstrom et al., *PCR Methods Applic. 1*:111-19, 1991) and walking PCR

(Parker et al., *Nucl. Acids. Res. 19*:3055-60, 1991). Other methods employing amplification may also be employed to obtain a full length cDNA sequence.

In certain instances, it is possible to obtain a full length cDNA sequence by analysis of sequences provided in an expressed sequence tag (EST) database, such as that available from GenBank. Searches for overlapping ESTs may generally be performed using well known programs (*e.g.,* NCBI BLAST searches), and such ESTs may be used to generate a contiguous full length sequence. Full length DNA sequences may also be obtained by analysis of genomic fragments.

In other embodiments of the invention, polynucleotide sequences or fragments thereof which encode polypeptides of the invention, or fusion proteins or functional equivalents thereof, may be used in recombinant DNA molecules to direct expression of a polypeptide in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences that encode substantially the same or a functionally equivalent amino acid sequence may be produced and these sequences may be used to clone and express a given polypeptide.

As will be understood by those of skill in the art, it may be advantageous in some instances to produce polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce a recombinant RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

Moreover, the polynucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter polypeptide encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. For example, DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. In addition, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation

patterns, change codon preference, produce splice variants, or introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences may be ligated to a heterologous sequence to encode a fusion protein. For example, to screen peptide libraries for inhibitors of polypeptide activity, it may be useful to encode a chimeric protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the polypeptide-encoding sequence and the heterologous protein sequence, so that the polypeptide may be cleaved and purified away from the heterologous moiety.

Sequences encoding a desired polypeptide may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M. H. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 215-223, Horn, T. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of a polypeptide, or a portion thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, J. Y. et al. (1995) *Science 269*:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer, Palo Alto, CA).

A newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, T. (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co., New York, N.Y.) or other comparable techniques available in the art. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure). Additionally, the amino acid sequence of a polypeptide, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a desired polypeptide, the nucleotide sequences encoding the polypeptide, or functional equivalents, may be inserted into appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled

in the art may be used to construct expression vectors containing sequences encoding a polypeptide of interest and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.

A variety of expression vector/host systems may be utilized to contain and express polynucleotide sequences. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" present in an expression vector are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the PBLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or PSPORT1 plasmid (Gibco BRL, Gaithersburg, MD) and the like may be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are generally preferred. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding a polypeptide, vectors based on SV40 or EBV may be advantageously used with an appropriate selectable marker.

In bacterial systems, any of a number of expression vectors may be selected depending upon the use intended for the expressed polypeptide. For example, when large quantities are needed, for example for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the sequence encoding the polypeptide of interest may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of .beta.-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S. M. Schuster (1989) *J. Biol. Chem. 264*:5503-5509); and the like. pGEX Vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, Saccharomyces cerevisiae, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) *Methods Enzymol.* 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding polypeptides may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J. 6*:307-311. Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) *EMBO J. 3*:1671-1680; Broglie, R. et al. (1984) *Science 224*:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ. 17*:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a

number of generally available reviews (see, for example, Hobbs, S. or Murry, L. E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, N.Y.; pp. 191-196).

An insect system may also be used to express a polypeptide of interest. For example, in one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The sequences encoding the polypeptide may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the polypeptide-encoding sequence will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, S. frugiperda cells or Trichoplusia larvae in which the polypeptide of interest may be expressed (Engelhard, E. K. et al. (1994) *Proc. Natl. Acad. Sci. 91* :3224-3227).

In mammalian host cells, a number of viral-based expression systems are generally available. For example, in cases where an adenovirus is used as an expression vector, sequences encoding a polypeptide of interest may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptide in infected host cells (Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci. 81*:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding a polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be

provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) *Results Probl. Cell Differ. 20*:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation. glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells such as CHO, COS, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is generally preferred. For example, cell lines which stably express a polynucleotide of interest may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) *Cell 11*:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1990) *Cell 22*:817-23) genes which can be employed in tk.sup.- or aprt.sup.- cells,

respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) *Proc. Natl. Acad. Sci. 77*:3567-70); npt, which confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin, F. et al (1981) *J. Mol. Biol. 150*:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, *supra*). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) *Proc. Natl. Acad. Sci. 85*:8047-51). The use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. et al. (1995) *Methods Mol. Biol. 55*:121-131).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding a polypeptide is inserted within a marker gene sequence, recombinant cells containing sequences can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a polypeptide-encoding sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells that contain and express a desired polynucleotide sequence may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include, for example, membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

A variety of protocols for detecting and measuring the expression of polynucleotide-encoded products, using either polyclonal or monoclonal antibodies specific

for the product are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a given polypeptide may be preferred for some applications, but a competitive binding assay may also be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul. Minn.) and Maddox, D. E. et al. (1983; *J. Exp. Med. 158*:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences, or any portions thereof may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits. Suitable reporter molecules or labels, which may be used include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with a polynucleotide sequence of interest may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides of the invention may be designed to contain signal sequences which direct secretion of the encoded polypeptide through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding a polypeptide of interest to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such

purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen. San Diego, Calif.) between the purification domain and the encoded polypeptide may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a polypeptide of interest and a nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography) as described in Porath, J. et al. (1992, *Prot. Exp. Purif. 3*:263-281) while the enterokinase cleavage site provides a means for purifying the desired polypeptide from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D. J. et al. (1993; *DNA Cell Biol. 12*:441-453).

In addition to recombinant production methods, polypeptides of the invention, and fragments thereof, may be produced by direct peptide synthesis using solid-phase techniques (Merrifield J. (1963) *J. Am. Chem. Soc. 85*:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Alternatively, various fragments may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

Antibody Compositions, Fragments Thereof and Other Binding Agents

According to another aspect, the present invention further provides binding agents, such as antibodies and antigen-binding fragments thereof, that exhibit immunological binding to a tumor polypeptide disclosed herein, or to a portion, variant or derivative thereof. An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunogically bind," and/or is "immunologically reactive" to a polypeptide of the invention if it reacts at a detectable level (within, for example, an ELISA

assay) with the polypeptide, and does not react detectably with unrelated polypeptides under similar conditions.

Immunological binding, as used in this context, generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off} /K_{on} enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant K_d. See, generally, Davies et al. (1990) Annual Rev. Biochem. 59:439-473.

An "antigen-binding site," or "binding portion" of an antibody refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each

of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

Binding agents may be further capable of differentiating between patients with and without a cancer, such as breast cancer, using the representative assays provided herein. For example, antibodies or other binding agents that bind to a tumor protein will preferably generate a signal indicating the presence of a cancer in at least about 20% of patients with the disease, more preferably at least about 30% of patients. Alternatively, or in addition, the antibody will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without the cancer. To determine whether a binding agent satisfies this requirement, biological samples (*e.g.*, blood, sera, sputum, urine and/or tumor biopsies) from patients with and without a cancer (as determined using standard clinical tests) may be assayed as described herein for the presence of polypeptides that bind to the binding agent. Preferably, a statistically significant number of samples with and without the disease will be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination to improve sensitivity.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome, with or without a peptide component, an RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior

immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* *6*:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

A number of therapeutically useful molecules are known in the art which comprise antigen-binding sites that are capable of exhibiting immunological binding properties of an antibody molecule. The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "$F(ab')_2$ " fragment which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using recombinant techniques known in the art. The Fv fragment includes a non-covalent $V_H::V_L$ heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) Proc. Nat. Acad. Sci. USA 69:2659-2662; Hochman et al. (1976) Biochem 15:2706-2710; and Ehrlich et al. (1980) Biochem 19:4091-4096.

A single chain Fv ("sFv") polypeptide is a covalently linked $V_H::V_L$ heterodimer which is expressed from a gene fusion including V_H- and V_L-encoding genes linked by a peptide-encoding linker. Huston et al. (1988) Proc. Nat. Acad. Sci. USA 85(16):5879-5883. A number of methods have been described to discern chemical structures for converting the naturally aggregated--but chemically separated--light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, *e.g.*, U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

Each of the above-described molecules includes a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain FR set which provide support to the CDRS and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An

antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRS. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures--regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

A number of "humanized" antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to human constant domains (Winter et al. (1991) Nature 349:293-299; Lobuglio et al. (1989) Proc. Nat. Acad. Sci. USA 86:4220-4224; Shaw et al. (1987) J Immunol. 138:4534-4538; and Brown et al. (1987) Cancer Res. 47:3577-3583), rodent CDRs grafted into a human supporting FR prior to fusion with an appropriate human antibody constant domain (Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536; and Jones et al. (1986) Nature 321:522-525), and rodent CDRs supported by recombinantly veneered rodent FRs (European Patent Publication No. 519,596, published

Dec. 23, 1992). These "humanized" molecules are designed to minimize unwanted immunological response toward rodent antihuman antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients.

As used herein, the terms "veneered FRs" and "recombinantly veneered FRs" refer to the selective replacement of FR residues from, *e.g.*, a rodent heavy or light chain V region, with human FR residues in order to provide a xenogeneic molecule comprising an antigen-binding site which retains substantially all of the native FR polypeptide folding structure. Veneering techniques are based on the understanding that the ligand binding characteristics of an antigen-binding site are determined primarily by the structure and relative disposition of the heavy and light chain CDR sets within the antigen-binding surface. Davies et al. (1990) Ann. Rev. Biochem. 59:439-473. Thus, antigen binding specificity can be preserved in a humanized antibody only wherein the CDR structures, their interaction with each other, and their interaction with the rest of the V region domains are carefully maintained. By using veneering techniques, exterior (*e.g.*, solvent-accessible) FR residues which are readily encountered by the immune system are selectively replaced with human residues to provide a hybrid molecule that comprises either a weakly immunogenic, or substantially non-immunogenic veneered surface.

The process of veneering makes use of the available sequence data for human antibody variable domains compiled by Kabat et al., in Sequences of Proteins of Immunological Interest, 4th ed., (U.S. Dept. of Health and Human Services, U.S. Government Printing Office, 1987), updates to the Kabat database, and other accessible U.S. and foreign databases (both nucleic acid and protein). Solvent accessibilities of V region amino acids can be deduced from the known three-dimensional structure for human and murine antibody fragments. There are two general steps in veneering a murine antigen-binding site. Initially, the FRs of the variable domains of an antibody molecule of interest are compared with corresponding FR sequences of human variable domains obtained from the above-identified sources. The most homologous human V regions are then compared residue by residue to corresponding murine amino acids. The residues in the murine FR

which differ from the human counterpart are replaced by the residues present in the human moiety using recombinant techniques well known in the art. Residue switching is only carried out with moieties which are at least partially exposed (solvent accessible), and care is exercised in the replacement of amino acid residues which may have a significant effect on the tertiary structure of V region domains, such as proline, glycine and charged amino acids.

In this manner, the resultant "veneered" murine antigen-binding sites are thus designed to retain the murine CDR residues, the residues substantially adjacent to the CDRs, the residues identified as buried or mostly buried (solvent inaccessible), the residues believed to participate in non-covalent (*e.g.*, electrostatic and hydrophobic) contacts between heavy and light chain domains, and the residues from conserved structural regions of the FRs which are believed to influence the "canonical" tertiary structures of the CDR loops. These design criteria are then used to prepare recombinant nucleotide sequences which combine the CDRs of both the heavy and light chain of a murine antigen-binding site into human-appearing FRs that can be used to transfect mammalian cells for the expression of recombinant human antibodies which exhibit the antigen specificity of the murine antibody molecule.

In another embodiment of the invention, monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, Pseudomonas exotoxin, Shigella toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl

group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody.

Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

<u>T Cell Compositions</u>

The present invention, in another aspect, provides T cells specific for a tumor polypeptide disclosed herein, or for a variant or derivative thereof. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood, or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system, such as the Isolex™ System, available from Nexell Therapeutics, Inc. (Irvine, CA; see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human mammals, cell lines or cultures.

T cells may be stimulated with a polypeptide, polynucleotide encoding a polypeptide and/or an antigen presenting cell (APC) that expresses such a polypeptide.

Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide of interest. Preferably, a tumor polypeptide or polynucleotide of the invention is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

T cells are considered to be specific for a polypeptide of the present invention if the T cells specifically proliferate, secrete cytokines or kill target cells coated with the polypeptide or expressing a gene encoding the polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res. 54*:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Contact with a tumor polypeptide (100 ng/ml - 100 μg/ml, preferably 200 ng/ml - 25 μg/ml) for 3 - 7 days will typically result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN-γ) is indicative of T cell activation (*see* Coligan et al., Current Protocols in Immunology, vol. 1, Wiley Interscience (Greene 1998)). T cells that have been activated in response to a tumor polypeptide, polynucleotide or polypeptide-expressing APC may be $CD4^+$ and/or $CD8^+$. Tumor polypeptide-specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient, a related donor or an unrelated donor, and are administered to the patient following stimulation and expansion.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in response to a tumor polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways.

For example, the T cells can be re-exposed to a tumor polypeptide, or a short peptide corresponding to an immunogenic portion of such a polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a tumor polypeptide. Alternatively, one or more T cells that proliferate in the presence of the tumor polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution.

Pharmaceutical Compositions

In additional embodiments, the present invention concerns formulation of one or more of the polynucleotide, polypeptide, T-cell and/or antibody compositions disclosed herein in pharmaceutically-acceptable carriers for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy.

It will be understood that, if desired, a composition as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, other proteins or polypeptides or various pharmaceutically-active agents. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA or DNA compositions.

Therefore, in another aspect of the present invention, pharmaceutical compositions are provided comprising one or more of the polynucleotide, polypeptide, antibody, and/or T-cell compositions described herein in combination with a physiologically acceptable carrier. In certain preferred embodiments, the pharmaceutical compositions of the invention comprise immunogenic polynucleotide and/or polypeptide compositions of the invention for use in prophylactic and theraputic vaccine applications. Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman,

eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Generally, such compositions will comprise one or more polynucleotide and/or polypeptide compositions of the present invention in combination with one or more immunostimulants.

It will be apparent that any of the pharmaceutical compositions described herein can contain pharmaceutically acceptable salts of the polynucleotides and polypeptides of the invention. Such salts can be prepared, for example, from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.*, salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium and magnesium salts).

In another embodiment, illustrative immunogenic compositions, *e.g.*, vaccine compositions, of the present invention comprise DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the polynucleotide may be administered within any of a variety of delivery systems known to those of ordinary skill in the art. Indeed, numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems 15*:143-198, 1998, and references cited therein. Appropriate polynucleotide expression systems will, of course, contain the necessary regulatory DNA regulatory sequences for expression in a patient (such as a suitable promoter and terminating signal). Alternatively, bacterial delivery systems may involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope.

Therefore, in certain embodiments, polynucleotides encoding immunogenic polypeptides described herein are introduced into suitable mammalian host cells for expression using any of a number of known viral-based systems. In one illustrative embodiment, retroviruses provide a convenient and effective platform for gene delivery systems. A selected nucleotide sequence encoding a polypeptide of the present invention can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to a subject. A number of illustrative retroviral systems have been described (*e.g.*, U.S. Pat. No. 5,219,740; Miller

and Rosman (1989) BioTechniques 7:980-990; Miller, A. D. (1990) Human Gene Therapy 1:5-14; Scarpa et al. (1991) Virology 180:849-852; Burns et al. (1993) Proc. Natl. Acad. Sci. USA 90:8033-8037; and Boris-Lawrie and Temin (1993) Cur. Opin. Genet. Develop. 3:102-109.

In addition, a number of illustrative adenovirus-based systems have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham (1986) J. Virol. 57:267-274; Bett et al. (1993) J. Virol. 67:5911-5921; Mittereder et al. (1994) Human Gene Therapy 5:717-729; Seth et al. (1994) J. Virol. 68:933-940; Barr et al. (1994) Gene Therapy 1:51-58; Berkner, K. L. (1988) BioTechniques 6:616-629; and Rich et al. (1993) Human Gene Therapy 4:461-476).

Various adeno-associated virus (AAV) vector systems have also been developed for polynucleotide delivery. AAV vectors can be readily constructed using techniques well known in the art. See, *e.g.*, U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski et al. (1988) Molec. Cell. Biol. 8:3988-3996; Vincent et al. (1990) Vaccines 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) Current Opinion in Biotechnology 3:533-539; Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158:97-129; Kotin, R. M. (1994) Human Gene Therapy 5:793-801; Shelling and Smith (1994) Gene Therapy 1:165-169; and Zhou et al. (1994) J. Exp. Med. 179:1867-1875.

Additional viral vectors useful for delivering the polynucleotides encoding polypeptides of the present invention by gene transfer include those derived from the pox family of viruses, such as vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel molecules can be constructed as follows. The DNA encoding a polypeptide is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the polypeptide of interest into the viral genome.

The resulting TK.sup.(-) recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

A vaccinia-based infection/transfection system can be conveniently used to provide for inducible, transient expression or coexpression of one or more polypeptides described herein in host cells of an organism. In this particular system, cells are first infected in vitro with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide or polynucleotides of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into polypeptide by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, *e.g.*, Elroy-Stein and Moss, Proc. Natl. Acad. Sci. USA (1990) 87:6743-6747; Fuerst et al. Proc. Natl. Acad. Sci. USA (1986) 83:8122-8126.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the coding sequences of interest. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an Avipox vector is particularly desirable in human and other mammalian species since members of the Avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant Avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, *e.g.*, WO 91/12882; WO 89/03429; and WO 92/03545.

Any of a number of alphavirus vectors can also be used for delivery of polynucleotide compositions of the present invention, such as those vectors described in U.S. Patent Nos. 5,843,723; 6,015,686; 6,008,035 and 6,015,694. Certain vectors based on Venezuelan Equine Encephalitis (VEE) can also be used, illustrative examples of which can be found in U.S. Patent Nos. 5,505,947 and 5,643,576.

Moreover, molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al. J. Biol. Chem. (1993) 268:6866-6869 and Wagner et al. Proc. Natl. Acad. Sci. USA (1992) 89:6099-6103, can also be used for gene delivery under the invention.

Additional illustrative information on these and other known viral-based delivery systems can be found, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; Kolls et al., *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; and Guzman et al., *Cir. Res. 73*:1202-1207, 1993.

In certain embodiments, a polynucleotide may be integrated into the genome of a target cell. This integration may be in the specific location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the polynucleotide may be stably maintained in the cell as a separate, episomal segment of DNA. Such polynucleotide segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. The manner in which the expression construct is delivered to a cell and where in the cell the polynucleotide remains is dependent on the type of expression construct employed.

In another embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In still another embodiment, a composition of the present invention can be delivered via a particle bombardment approach, many of which have been described. In

one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide or polypeptide particles, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

According to another embodiment, the pharmaceutical compositions described herein will comprise one or more immunostimulants in addition to the immunogenic polynucleotide, polypeptide, antibody, T-cell and/or APC compositions of this invention. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres;

monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Within certain embodiments of the invention, the adjuvant composition is preferably one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science 273*:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins . Other preferred formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamelar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol^{R} to increase viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

In one preferred embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL® adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. Another particularly preferred adjuvant formulation employing QS21, 3D-MPL® adjuvant and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 is disclosed in WO 00/09159. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*, SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Enhanzyn®) (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, the disclosures of which are

incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants such as those described in WO 99/52549A1.

Other preferred adjuvants include adjuvant molecules of the general formula (I): $HO(CH_2CH_2O)_n$-A-R,

wherein, n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-steoryl ether, polyoxyethylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12^{th} edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

According to another embodiment of this invention, an immunogenic composition described herein is delivered to a host via antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, *Nature 392*:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman and Levy, *Ann. Rev. Med. 50*:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel et al., *Nature Med. 4*:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNFα to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNFα, CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface

molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.*, CD54 and CD11) and costimulatory molecules (*e.g.*, CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide of the invention (or portion or other variant thereof) such that the encoded polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a pharmaceutical composition comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., *Immunology and cell Biology* 75:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the tumor polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (*e.g.*, vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (*e.g.*, a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will typically vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, mucosal, intravenous, intracranial, intraperitoneal, subcutaneous and intramuscular administration.

Carriers for use within such pharmaceutical compositions are biocompatible, and may also be biodegradable. In certain embodiments, the formulation preferably provides a relatively constant level of active component release. In other embodiments,

however, a more rapid rate of release immediately upon administration may be desired. The formulation of such compositions is well within the level of ordinary skill in the art using known techniques. Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.,* a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.,* U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

In another illustrative embodiment, biodegradable microspheres (*e.g.,* polylactate polyglycolate) are employed as carriers for the compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems. such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Patent No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

The pharmaceutical compositions of the invention will often further comprise one or more buffers (*e.g.,* neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.,* glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically sealed in such a way to preserve the sterility and stability of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, is well known in the art, some of which are briefly discussed below for general purposes of illustration.

In certain applications, the pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (see, for example, Mathiowitz *et al.*, Nature 1997 Mar 27;386(6623):410-4; Hwang *et al.*, Crit Rev Ther Drug Carrier Syst 1998;15(3):243-84; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451). Tablets, troches, pills, capsules and the like may also contain any of a variety of additional components, for example, a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be

present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations will contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. Such approaches are well known to the skilled artisan, some of which are further described, for example, in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363. In certain embodiments, solutions of the active compounds as free base

or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally will contain a preservative to prevent the growth of microorganisms.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or

injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will of course preferably meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

In another embodiment of the invention, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described, *e.g.,* in U. S. Patent 5,756,353 and U. S. Patent

5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively, compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, J Biol Chem. 1990 Sep 25;265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, various drugs, radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore, he use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.*, Crit Rev Ther Drug Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar;45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2;50(1-3):31-40; and U. S. Patent 5,145,684.

Cancer Therapeutic Methods

In further aspects of the present invention, the pharmaceutical compositions described herein may be used for the treatment of cancer, particularly for the immunotherapy of breast cancer. Within such methods, the pharmaceutical compositions described herein are administered to a patient, typically a warm-blooded animal, preferably a human. A patient may or may not be afflicted with cancer. Accordingly, the above pharmaceutical compositions may be used to prevent the development of a cancer or to treat a patient afflicted with a cancer. Pharmaceutical compositions and vaccines may be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs. As discussed above, administration of the pharmaceutical compositions may be by any suitable method, including administration by intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, intradermal, anal, vaginal, topical and oral routes.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous host

immune system to react against tumors with the administration of immune response-modifying agents (such as polypeptides and polynucleotides as provided herein).

Within other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T cells as discussed above, T lymphocytes (such as $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and macrophages) expressing a polypeptide provided herein. T cell receptors and antibody receptors specific for the polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The polypeptides provided herein may also be used to generate antibodies or anti-idiotypic antibodies (as described above and in U.S. Patent No. 4,918,164) for passive immunotherapy.

Effector cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions typically use intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive polypeptides as provided herein may be used to rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage, monocyte, fibroblast and/or B cells, may be pulsed with immunoreactive polypeptides or transfected with one or more polynucleotides using standard techniques well known in the art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*.

Studies have shown that cultured effector cells can be induced to grow in vivo and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (*see*, for example, Cheever et al., *Immunological Reviews 157*:177, 1997).

Alternatively, a vector expressing a polypeptide recited herein may be introduced into antigen presenting cells taken from a patient and clonally propagated *ex vivo* for transplant back into the same patient. Transfected cells may be reintroduced into the patient using any means known in the art, preferably in sterile form by intravenous, intracavitary, intraperitoneal or intratumor administration.

Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response, and is at least 10-50% above the basal (*i.e.*, untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 25 μg to 5 mg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to a tumor protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

Cancer Detection and Diagnostic Compositions, Methods and Kits

In general, a cancer may be detected in a patient based on the presence of one or more breast tumor proteins and/or polynucleotides encoding such proteins in a biological sample (for example, blood, sera, sputum urine and/or tumor biopsies) obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of a cancer such as breast cancer. In addition, such proteins may be useful for the detection of other cancers. The binding agents provided herein generally permit detection of the level of antigen that binds to the agent in the biological sample. Polynucleotide primers and probes may be used to detect the level of mRNA encoding a tumor protein, which is also indicative of the presence or absence of a cancer. In general, a breast tumor sequence should be present at a level that is at least three fold higher in tumor tissue than in normal tissue

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. *See*, *e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

In a preferred embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length breast tumor proteins and polypeptide portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in the art to which the tumor protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1

hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μg, and preferably about 100 ng to about 1 μg, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see*, *e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with breast cancer. Preferably, the contact time is

sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, such as breast cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of a cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for the cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve,

according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of

antibody immobilized on the membrane ranges from about 25 ng to about 1μg, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the tumor proteins or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use tumor polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such tumor protein specific antibodies may correlate with the presence of a cancer.

A cancer may also, or alternatively, be detected based on the presence of T cells that specifically react with a tumor protein in a biological sample. Within certain methods, a biological sample comprising $CD4^+$ and/or $CD8^+$ T cells isolated from a patient is incubated with a tumor polypeptide, a polynucleotide encoding such a polypeptide and/or an APC that expresses at least an immunogenic portion of such a polypeptide, and the presence or absence of specific activation of the T cells is detected. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with polypeptide (*e.g.*, 5 - 25 μg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of tumor polypeptide to serve as a control. For $CD4^+$ T cells, activation is preferably detected by evaluating proliferation of the T cells. For $CD8^+$ T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer in the patient.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding a tumor protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a tumor cDNA derived from a biological sample, wherein at

least one of the oligonucleotide primers is specific for (*i.e.*, hybridizes to) a polynucleotide encoding the tumor protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes that specifically hybridize to a polynucleotide encoding a tumor protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the tumor protein in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a tumor protein of the invention that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes hybridize to a polynucleotide encoding a polypeptide described herein under moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In a preferred embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule having a sequence as disclosed herein. Techniques for both PCR based assays and hybridization assays are well known in the art (*see*, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, *51*:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a biological sample, such as biopsy tissue, and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in

expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another embodiment, the compositions described herein may be used as markers for the progression of cancer. In this embodiment, assays as described above for the diagnosis of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) or polynucleotide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom the level of polypeptide or polynucleotide detected increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide or polynucleotide either remains constant or decreases with time.

Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

As noted above, to improve sensitivity, multiple tumor protein markers may be assayed within a given sample. It will be apparent that binding agents specific for different proteins provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of tumor protein markers may be based on routine experiments to determine combinations that results in optimal sensitivity. In addition, or alternatively, assays for tumor proteins provided herein may be combined with assays for other known tumor antigens.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a tumor protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or

more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a tumor protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a tumor protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a tumor protein.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

ISOLATION AND CHARACTERIZATION OF BREAST TUMOR POLYPEPTIDES

This Example describes the isolation of breast tumor polypeptides from a breast tumor cDNA library.

A cDNA subtraction library containing cDNA from breast tumor subtracted with normal breast cDNA was constructed as follows. Total RNA was extracted from primary tissues using Trizol reagent (Gibco BRL Life Technologies, Gaithersburg, MD) as described by the manufacturer. The polyA+ RNA was purified using an oligo(dT) cellulose column according to standard protocols. First strand cDNA was synthesized using the primer supplied in a Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA). The driver DNA consisted of cDNAs from two normal breast tissues with the tester cDNA being from three primary breast tumors. Double-stranded cDNA was synthesized for both tester and driver, and digested with a combination of endonucleases (MluI, MscI, PvuII, SalI and StuI) which recognize six base pairs DNA. This modification increased the

average cDNA size dramatically compared with cDNAs generated according to the protocol of Clontech (Palo Alto, CA). The digested tester cDNAs were ligated to two different adaptors and the subtraction was performed according to Clontech's protocol. The subtracted cDNAs were subjected to two rounds of PCR amplification, following the manufacturer's protocol. The resulting PCR products were subcloned into the TA cloning vector, pCRII (Invitrogen, San Diego, CA) and transformed into ElectroMax *E. coli* DH10B cells (Gibco BRL Life, Technologies) by electroporation. DNA was isolated from independent clones and sequenced using a Perkin Elmer/Applied Biosystems Division (Foster City, CA) Automated Sequencer Model 373A.

Sixty-three distinct cDNA clones were found in the subtracted breast tumor-specific cDNA library. The determined one strand (5' or 3') cDNA sequences for the clones are provided in SEQ ID NO:1-61, 72 and 73, respectively. Comparison of these cDNA sequences with known sequences in the gene bank using the EMBL and GenBank databases (Release 97) revealed no significant homologies to the sequences provided in SEQ ID NO:14, 21, 22, 27, 29, 30, 32, 38, 44, 45, 53, 57, 72 and 73. The sequences of SEQ ID NO: 1, 3, 16, 17, 34, 48, 60 and 61 were found to represent known human genes. The sequences of SEQ ID NO:2, 4, 23, 39 and 50 were found to show some similarity to previously identified non-human genes. The remaining clones (SEQ ID NO:5-13, 15, 18-20, 24-26, 28, 31, 33, 35-37, 40-43, 46, 47, 49, 51, 52, 54-56, 58 and 59) were found to show at least some degree of homology to previously identified expressed sequence tags (ESTs).

Further studies resulted in the isolation of the full-length cDNA sequence for the clone of SEQ ID NO:57 (referred to as B718P). By computer analysis, the full-length sequence was found to contain a putative transmembrane domain at amino acids 137-158. The full-length cDNA sequence of B718P is provided in SEQ ID NO:504, with the cDNA sequence of the open reading frame including stop codon being provided in SEQ ID NO:505 and the cDNA sequence of the open reading frame without stop codon being provided in SEQ ID NO:506. The full-length amino acid sequence of B718P is provided is

SEQ ID NO:507. SEQ ID NO:508 represents amino acids 1-158 of B718P, and SEQ ID NO:509 represents amino acids 159-243 of B718P.

To determine mRNA expression levels of the isolated cDNA clones, cDNA clones from the breast subtraction described above were randomly picked and colony PCR amplified. Their mRNA expression levels in breast tumor, normal breast and various other normal tissues were determined using microarray technology (Synteni, Palo Alto, CA). Briefly, the PCR amplification products were arrayed onto slides in an array format, with each product occupying a unique location in the array. mRNA was extracted from the tissue sample to be tested, reverse transcribed, and fluorescent-labeled cDNA probes were generated. The microarrays were probed with the labeled cDNA probes, the slides scanned and fluorescence intensity was measured. Data was analyzed using Synteni provided GEMTOOLS Software. Of the seventeen cDNA clones examined, those of SEQ ID NO:40, 46, 59 and 73 were found to be over-expressed in breast tumor and expressed at low levels in all normal tissues tested (breast, PBMC, colon, fetal tissue, salivary gland, bone marrow, lung, pancreas, large intestine, spinal cord, adrenal gland, kidney, pancreas, liver, stomach, skeletal muscle, heart, small intestine, skin, brain and human mammary epithelial cells). The clones of SEQ ID NO:41 and 48 were found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested, with the exception of bone marrow. The clone of SEQ ID NO:42 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested except bone marrow and spinal cord. The clone of SEQ ID NO:43 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of spinal cord, heart and small intestine. The clone of SEQ ID NO:51 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of large intestine. The clone of SEQ ID NO:54 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of PBMC, stomach and small intestine. The clone of SEQ ID NO:56 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of large and small intestine, human mammary epithelia cells and SCID mouse-passaged breast tumor. The clone of SEQ ID NO:60 was

found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of spinal cord and heart. The clone of SEQ ID NO:61 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of small intestine. The clone of SEQ ID NO:72 was found to be over-expressed in breast tumor and expressed at low levels in all other tissues tested with the exception of colon and salivary gland.

The results of a Northern blot analysis of the clone SYN18C6 (SEQ ID NO:40) are shown in Fig. 1. A predicted protein sequence encoded by SYN18C6 is provided in SEQ ID NO:62.

Additional cDNA clones that are over-expressed in breast tumor tissue were isolated from breast cDNA subtraction libraries as follows. Breast subtraction libraries were prepared, as described above, by PCR-based subtraction employing pools of breast tumor cDNA as the tester and pools of either normal breast cDNA or cDNA from other normal tissues as the driver. cDNA clones from breast subtraction were randomly picked and colony PCR amplified and their mRNA expression levels in breast tumor, normal breast and various other normal tissues were determined using the microarray technology described above. Twenty-four distinct cDNA clones were found to be over-expressed in breast tumor and expressed at low levels in all normal tissues tested (breast, brain, liver, pancreas, lung, salivary gland, stomach, colon, kidney, bone marrow, skeletal muscle, PBMC, heart, small intestine, adrenal gland, spinal cord, large intestine and skin). The determined cDNA sequences for these clones are provided in SEQ ID NO:63-87. Comparison of the sequences of SEQ ID NO:74-87 with those in the gene bank as described above, revealed homology to previously identified human genes. No significant homologies were found to the sequences of SEQ ID NO:63-73.

Three DNA isoforms for the clone B726P (partial sequence provided in SEQ ID NO:71) were isolated as follows. A radioactive probe was synthesized from B726P by excising B726P DNA from a pT7Blue vector (Novagen) by a BamHI/XbaI restriction digest and using the resulting DNA as the template in a single-stranded PCR in the presence of [α-32P]dCTP. The sequence of the primer employed for this PCR is

provided in SEQ ID NO:177. The resulting radioactive probe was used to probe a directional cDNA library and a random-primed cDNA library made using RNA isolated from breast tumors. Eighty-five clones were identified, excised, purified and sequenced. Of these 85 clones, three were found to each contain a significant open reading frame. The determined cDNA sequence of the isoform B726P-20 is provided in SEQ ID NO:175, with the corresponding predicted amino acid sequence being provided in SEQ ID NO:176. The determined cDNA sequence of the isoform B726P-74 is provided in SEQ ID NO:178, with the corresponding predicted amino acid sequence being provided in SEQ ID NO:179. The determined cDNA sequence of the isoform B726P-79 is provided in SEQ ID NO:180, with the corresponding predicted amino acid sequence being provided in SEQ ID NO:181.

Efforts to obtain a full-length clone of B726P using standard techniques led to the isolation of five additional clones that represent additional 5' sequence of B726P. These clones appear to be alternative splice forms of the same gene. The determined cDNA sequences of these clones are provided in SEQ ID NO:464-468, with the predicted amino acid sequences encoded by SEQ ID NO: 464-467 being provided in SEQ ID NO:470-473, respectively. Using standard computer techniques, a 3,681 bp consensus DNA sequence (SEQ ID NO:463) was created that contains two large open reading frames. The downstream ORF encodes the amino acid sequence of SEQ ID NO:176. The predicted amino acid sequence encoded by the upstream ORF is provided in SEQ ID NO:469. Subsequent studies led to the isolation of an additional splice form of B726P that has 184 bp insert relative to the other forms. This 184 bp insert causes a frameshift that brings the down stream and upstream ORFs together into a single ORF that is 1002 aa in length. The determined cDNA sequence of this alternative splice form is disclosed in SEQ ID NO:474, with the corresponding amino acid sequence being provided in SEQ ID NO:475.

Comparison of the cDNA sequence of SEQ ID NO:63 (referred to as B723P) with the sequences in the GeneSeq™ DNA database showed matches to 5 DNA sequences (Accession nos. A26456, A37144, A26424, V84525 and T22133), 4 of which appear to represent the full-length sequence of the gene. Three of these sequences encode a 243 amino acid open reading frame (ORF), while one of the DNA sequences (Accession

no. A37144) contains an extra C at position 35, resulting in a 278 amino acid ORF. The open reading frame, including stop codon, of the first variant of B723P (referred to as B723P-short) is provided in SEQ ID NO:510, with the open reading frame without stop codon being provided in SEQ ID NO:511. The open reading frame, including stop codon, of the second variant of B723P (referred to as B723P-long) is provided in SEQ ID NO:512, with the open reading frame without stop codon being provided in SEQ ID NO:513. The amino acid sequences of B723P-short and B723P-long are provided in SEQ ID NO:514 and 515, respectively. Computer analysis of these sequences demonstrated the presence of putative transmembrane domains at amino acids 233-252 of the B723P-long ORF and amino acids 198-217 of the B723P-short ORF. SEQ ID NO:516, 518 and 519 represent amino acids 1-197, 198-243 and 218-243, respectively of B723P-short. SEQ ID NO:517 represents amino acids 1-232 of B723P-long.

Further isolation of individual clones that are over-expressed in breast tumor tissue was conducted using cDNA subtraction library techniques described above. In particular, a cDNA subtraction library containing cDNA from breast tumors subtracted with five other normal human tissue cDNAs (brain, liver, PBMC, pancreas and normal breast) was utilized in this screening. From the original subtraction, one hundred seventy seven clones were selected to be further characterized by DNA sequencing and microarray analysis. Microarray analysis demonstrated that the sequences in SEQ ID NO:182-251 and 479 were 2 or more fold over-expressed in human breast tumor tissues over normal human tissues. No significant homologies were found for nineteen of these clones, including, SEQ ID NO:185, 186, 194, 199, 205, 208, 211, 214-216, 219, 222, 226, 232, 236, 240, 241, 245, 246 and 479, with the exception of some previously identified expressed sequence tags (ESTs). The remaining clones share some homology to previously identified genes, specifically SEQ ID NO:181-184, 187-193, 195-198, 200-204, 206, 207, 209, 210, 212, 213, 217, 218, 220, 221, 223-225, 227-231, 233-235, 237-239, 242-244 and 247-251.

One of the cDNA clones isolated by PCR subtraction as described above (SEQ ID NO:476; referred to as B720P) which was shown by microarray to be over-expressed in breast tumor tissues, was found to be identical to a known keratin gene. The

full-length cDNA sequence of the known keratin gene is provided in SEQ ID NO:477, with the corresponding amino acid sequence being provided in SEQ ID NO:478. Primers were generated based on the sequence of SEQ ID NO:477 and used to clone full-length cDNA from mRNA which was obtained from total RNA showing high expression of B720P in real-time PCR analysis. Products were then cloned and sequenced. The determined full-length cDNA sequence for B720P is provided in SEQ ID NO:484, with the corresponding amino acid sequence being provided in SEQ ID NO:485.

In further studies, a truncated form of B720P (referred to as B720P-tr) was identified in breast carcinomas. This antigen was cloned from mRNA derived from total breast tumor RNA that showed high expression of B720P-tr in real-time PCR analysis. mRNA was used to generate a pool of cDNA which was then used as a template to amplify the cDNA corresponding to B720P-tr by PCR. The determined cDNA sequence for B720P-tr is provided in SEQ ID NO:486. B720P-tr has an ORF of 708 base pairs which encodes a 236 amino acid protein (SEQ ID NO:487). The size of the transcript was confirmed by northern analysis.

Of the seventy clones showing over-expression in breast tumor tissues, fifteen demonstrated particularly good expression levels in breast tumor over normal human tissues. The following eleven clones did not show any significant homology to any known genes. Clone 19463.1 (SEQ ID NO:185) was over-expressed in the majority of breast tumors and also in the SCID breast tumors tested (refer to Example 2); additionally, over-expression was found in a majority of normal breast tissues. Clone 19483.1 (SEQ ID NO:216) was over-expressed in a few breast tumors, with no over-expression in any normal tissues tested. Clone 19470.1 (SEQ ID NO:219) was found to be slightly over-expressed in some breast tumors. Clone 19468.1 (SEQ ID NO:222) was found to be slightly over-expressed in the majority of breast tumors tested. Clone 19505.1 (SEQ ID NO:226) was found to be slightly over-expressed in 50% of breast tumors, as well as in SCID tumor tissues, with some degree of over-expression in found in normal breast. Clone 1509.1 (SEQ ID NO:232) was found to be over-expressed in very few breast tumors, but with a certain degree of over-expression in metastatic breast tumor tissues, as well as no

significant over-expression found in normal tissues. Clone 19513.1 (SEQ ID NO:236) was shown to be slightly over-expressed in few breast tumors, with no significant over-expression levels found in normal tissues. Clone 19575.1 (SEQ ID NO:240) showed low level over-expression in some breast tumors and also in normal breast. Clone 19560.1 (SEQ ID NO:241) was over-expressed in 50% of breast tumors tested, as well as in some normal breast tissues. Clone 19583.1 (SEQ ID NO:245) was slightly over-expressed in some breast tumors, with very low levels of over-expression found in normal tissues. Clone 19587.1 (SEQ ID NO:246) showed low level over-expression in some breast tumors and no significant over-expression in normal tissues.

Clone 19520.1 (SEQ ID NO:233), showing homology to clone 102D24 on chromosome 11q13.31, was found to be over-expressed in breast tumors and in SCID tumors. Clone 19517.1 (SEQ ID NO:237), showing homology to human PAC 128M19 clone, was found to be slightly over-expressed in the majority of breast tumors tested. Clone 19392.2 (SEQ ID NO:247), showing homology to human chromosome 17, was shown to be over-expressed in 50% of breast tumors tested. Clone 19399.2 (SEQ ID NO:250), showing homology to human Xp22 BAC GSHB-184P14, was shown to be slightly over-expressed in a limited number of breast tumors tested.

In subsequent studies, 64 individual clones were isolated from a subtracted cDNA library containing cDNA from a pool of breast tumors subtracted with cDNA from five normal tissues (brain, liver, PBMC, pancreas and normal breast). The subtracted cDNA library was prepared as described above with the following modification. A combination of five six-base cutters (MluI, MscI, PvuII, SalI and StuI) was used to digest the cDNA instead of RsaI. This resulted in an increase in the average insert size from 300 bp to 600 bp. The 64 isolated clones were colony PCR amplified and their mRNA expression levels in breast tumor tissue, normal breast and various other normal tissues were examined by microarray technology as described above. The determined cDNA sequences of 11 clones which were found to be over-expressed in breast tumor tissue are provided in SEQ ID NO:405-415. Comparison of these sequences to those in the public database, as outlined above, revealed homologies between the sequences of SEQ ID

NO:408, 411, 413 and 414 and previously isolated ESTs. The sequences of SEQ ID NO:405-407, 409, 410, 412 and 415 were found to show some homology to previously identified sequences.

In further studies, a subtracted cDNA library was prepared from cDNA from metastatic breast tumors subtracted with a pool of cDNA from five normal tissues (breast, brain, lung, pancreas and PBMC) using the PCR-subtraction protocol of Clontech, described above. The determined cDNA sequences of 90 clones isolated from this library are provided in SEQ ID NO:316-404. Comparison of these sequences with those in the public database, as described above, revealed no significant homologies to the sequence of SEQ ID NO:366. The sequences of SEQ ID NO:321-325, 343, 354, 368, 369, 377, 382, 385, 389, 395, 397 and 400 were found to show some homology to previously isolated ESTs. The remaining sequences were found to show homology to previously identified gene sequences.

In yet further studies, a subtracted cDNA library (referred to as 2BT) was prepared from cDNA from breast tumors subtracted with a pool of cDNA from six normal tissues (liver, brain, stomach, small intestine, kidney and heart) using the PCR-subtraction protocol of Clontech, described above. cDNA clones isolated from this subtraction were subjected to DNA microarray analysis as described above and the resulting data subjected to four modified Gemtools analyses. The first analysis compared 28 breast tumors with 28 non-breast normal tissues. A mean over-expression of at least 2.1 fold was used as a selection cut-off. The second analysis compared 6 metastatic breast tumors with 29 non-breast normal tissues. A mean over-expression of at least 2.5 fold was used as a cut-off. The third and fourth analyses compared 2 early SCID mouse-passaged with 2 late SCID mouse-passaged tumors. A mean over-expression in the early or late passaged tumors of 2.0 fold or greater was used as a cut-off. In addition, a visual analysis was performed on the microarray data for the 2BT clones. The determined cDNA sequences of 13 clones identified in the visual analysis are provided in SEQ ID NO:427-439. The determined cDNA sequences of 22 clones identified using the modified Gemtools analysis are provided

in SEQ ID NO:440-462, wherein SEQ ID NO:453 and 454 represent two partial, non-overlapping, sequences of the same clone.

Comparison of the clone sequences of SEQ ID NO:436 and 437 (referred to as 263G6 and 262B2) with those in the public databases, as described above, revealed no significant homologies to previously identified sequences. The sequences of SEQ ID NO:427, 429, 431, 435, 438, 441, 443, 444, 445, 446, 450, 453 and 454 (referred to as 266B4, 266G3, 264B4, 263G1, 262B6, 2BT2-34, 2BT1-77, 2BT1-62, 2BT1-60,61, 2BT1-59, 2BT1-52 and 2BT1-40, respectively) showed some homology to previously isolated expressed sequences tags (ESTs). The sequences of SEQ ID NO:428, 430, 432, 433, 434, 439, 440, 442, 447, 448, 449, 451, 452 and 455-462 (referred to as clones 22892, 22890, 22883, 22882, 22880, 22869, 21374, 21349, 21093, 21091, 21089, 21085, 21084, 21063, 21062, 21060, 21053, 21050, 21036, 21037 and 21048, respectively), showed some homology to gene sequences previously identified in humans.

EXAMPLE 2

ISOLATION AND CHARACTERIZATION OF BREAST TUMOR POLYPEPTIDES OBTAINED BY PCR-BASED SUBTRACTION USING SCID-PASSAGED TUMOR RNA

Human breast tumor antigens were obtained by PCR-based subtraction using SCID mouse passaged breast tumor RNA as follows. Human breast tumor was implanted in SCID mice and harvested on the first or sixth serial passage, as described in Patent Application Serial No. 08/556,659 filed 11/13/95, U.S. Patent No. 5,986,170. Genes found to be differentially expressed between early and late passage SCID tumor may be stage specific and therefore useful in therapeutic and diagnostic applications. Total RNA was prepared from snap frozen SCID passaged human breast tumor from both the first and sixth passage.

PCR-based subtraction was performed essentially as described above. In the first subtraction (referred to as T9), RNA from first passage tumor was subtracted from sixth passage tumor RNA to identify more aggressive, later passage-specific antigens. Of the 64 clones isolated and sequenced from this subtraction, no significant homologies were

found to 30 of these clones, hereinafter referred to as: 13053, 13057, 13059, 13065, 13067, 13068, 13071-13073, 13075, 13078, 13079, 13081, 13082, 13092, 13097, 13101, 13102, 13131, 13133, 13119, 13135, 13139, 13140, 13146-13149, and 13151, with the exception of some previously identified expressed sequence tags (ESTs). The determined cDNA sequences for these clones are provided in SEQ ID NO:88-116, respectively. The isolated cDNA sequences of SEQ ID NO:117-140 showed homology to known genes.

In a second PCR-based subtraction, RNA from sixth passage tumor was subtracted from first passage tumor RNA to identify antigens down-regulated over multiple passages. Of the 36 clones isolated and sequenced, no significant homologies were found to nineteen of these clones, hereinafter referred to as: 14376, 14377, 14383, 14384, 14387, 14392, 14394, 14398, 14401, 14402, 14405, 14409, 14412, 14414-14416, 14419, 14426, and 14427, with the exception of some previously identified expressed sequence tags (ESTs). The determined cDNA sequences for these clones are provided in SEQ ID NO:141-159, respectively. The isolated cDNA sequences of SEQ ID NO: 160-174 were found to show homology to previously known genes.

Further analysis of human breast tumor antigens through PCR-based subtraction using first and sixth passage SCID tumor RNA was performed. Sixty three clones were found to be differentially expressed by a two or more fold margin, as determined by microarray analysis, i.e., higher expression in early passage tumor over late passage tumor, or vice versa.. Seventeen of these clones showed no significant homology to any known genes, although some degree of homology with previously identified expressed sequence tags (ESTs) was found, hereinafter referred to as 20266, 20270, 20274, 20276, 20277, 20280, 20281, 20294, 20303, 20310, 20336, 20341, 20941, 20954, 20961, 20965 and 20975 (SEQ ID NO:252-268, respectively). The remaining clones were found to share some degree of homology to known genes, which are identified in the Brief Description of the Drawings and Sequence Identifiers section above, hereinafter referred to as 20261, 20262, 20265, 20267, 20268, 20271, 20272, 20273, 20278, 20279, 20293, 20300, 20305, 20306, 20307, 20313, 20317, 20318, 20320, 20321, 20322, 20326, 20333, 20335, 20337, 20338, 20340, 20938, 20939, 20940, 20942, 20943, 20944, 20946, 20947,

20948, 20949, 20950, 20951, 20952, 20957, 20959, 20966, 20976, 20977 and 20978. The determined cDNA sequences for these clones are provided in SEQ ID NO:269-314, respectively.

The clones 20310, 20281, 20262, 20280, 20303, 20336, 20270, 20341, 20326 and 20977 (also referred to as B820P, B821P, B822P, B823P, B824P, B825P, B826P, B827P, B828P and B829P, respectively) were selected for further analysis based on the results obtained with microarray analysis. Specifically, microarray data analysis indicated at least two- to three-fold overexpression of these clones in breast tumor RNA compared to normal tissues tested. Subsequent studies led to the determination of the complete insert sequence for the clones B820P, B821P, B822P, B823P, B824P, B825P, B826P, B827P, B828P and B829P. These extended cDNA sequences are provided in SEQ ID NO:416-426, respectively.

EXAMPLE 3

SYNTHESIS OF POLYPEPTIDES

Polypeptides may be synthesized on an Perkin Elmer/Applied Biosystems Division 430A peptide synthesizer using FMOC chemistry with HPTU (O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly sequence may be attached to the amino terminus of the peptide to provide a method of conjugation, binding to an immobilized surface, or labeling of the peptide. Cleavage of the peptides from the solid support may be carried out using the following cleavage mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After cleaving for 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The peptide pellets may then be dissolved in water containing 0.1% trifluoroacetic acid (TFA) and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of 0%-60% acetonitrile (containing 0.1% TFA) in water (containing 0.1% TFA) may be used to elute the peptides. Following lyophilization of the pure fractions, the peptides may be characterized using electrospray or other types of mass spectrometry and by amino acid analysis.

EXAMPLE 4

ELICITATION OF BREAST ANTIGEN-SPECIFIC CTL RESPONSES IN HUMAN BLOOD

This Example illustrates the ability of the breast-specific antigen B726P to elicit a cytotoxic T lymphocyte (CTL) response in peripheral blood lymphocytes from normal humans.

Autologous dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of a normal donor by growth for five days in RPMI medium containing 10% human serum, 30 ng/ml GM-CSF and 30 ng/ml IL-4. Following five days of culture, DC were infected overnight with adenovirus expressing recombinant B726P (downstream ORF; SEQ ID NO:176) at an M.O.I. of 2.5 and matured for 8 hours by the addition of 2 micrograms/ml CD40 ligand. CD8 positive cells were enriched for by the depletion of CD4 and CD14-positive cells. Priming cultures were initiated in individual wells of several 96-well plates with the cytokines IL-6 and IL-12. These cultures were restimulated in the presence of IL-2 using autologous fibroblasts treated with IFN-gamma and transduced with B726P and CD80. Following three stimulation cycles, the presence of B726P-specific CTL activity was assessed in IFN-gamma Elispot assays (Lalvani et al., *J. Exp. Med. 186*:859-865, 1997) using IFN-gamma treated autologous fibroblasts transduced to express either B726P or an irrelevant, control, antigen as antigen presenting cells (APC). Of approximately 96 lines, one line (referred to as 6-2B) was identified that appeared to specifically recognize B726P-transduced APC but not control antigen-transduced APC. This microculture was cloned using standard protocols. B726P-specific CTL were identified by Elispot analysis and expanded for further analysis. These CTL clones were demonstrated to recognize B726P-expressing fibroblasts, but not the control antigen MART-1, using chromium-51 release assays. Furthermore, using a panel of allogeneic fibroblasts transduced with B726P in antibody blocking assays, the HLA restriction element for these B726P-specific CTL was identified as HLA-B*1501.

In order to define more accurately the location of the epitope recognized by the B726P-specific CTL clones, a deletion construct comprising only the N-terminal half

(a.a. 1-129) of B726P (referred to as B726Pdelta3') was constructed in the pBIB retroviral expression plasmid. This plasmid, as well as other plasmids containing B726P, were transfected into COS-7 cells either alone or in combination with a plasmid expressing HLA-B*1501. Aproximately 48 hours after transfection, a B726P-specific CTL clone (1-9B) was added at approximately 10^4 cells per well. The cells were harvested the next day and the amount of IFN-gamma released was measured by ELISA. The CTL responded above background (EGFP) to COS-7 cells that had been transfected with both B726P and HLA-B*1501. There was no response above background to COS-7 cells that had been transfected with either B726P or HLA-B*1501 alone. Importantly, a higher response was seen with COS-7 cells that had been transfected with both HLA-B*1501 and B726Pdelta3'. This result indicated that the epitope was likely to be located in the N-terminal region (a.a. 1-129) of B726P. This region was examined and amino acid sequences that corresponded to the HLA-B*1501 peptide binding motif (*J. Immunol.*1999,*162*:7277-84) were identified and synthesized. These peptides were pulsed at 10 ug/ml onto autologous B-LCL overnight. The next day, the cells were washed and the ability of the cells to stimulate the B726P-specific CTL clone 1-9B was assayed in a IFN-gamma ELISPOT assay. Of the eleven peptides tested, only one peptide, having the amino acid sequence SLTKRASQY (a.a. 76-84 of B726P; SEQ ID NO: 488) was recognized by the CTL clone. This result identifies this peptide as being a naturally-processed epitope recognized by this B726P-specific CTL clone.

In further studies, a panel of breast tumor cell lines obtained from the American Type Culture Collection (Manassas, VA), was analyzed using real time PCR to determine their B726P message level. The cell line that expressed the highest level of B726P (referred to as HTB21) and a line that expressed no B726P (referred to as HTB132) were transduced with HLA-B*1501. These cell lines were grown up and analyzed using FACS to determine their B1501 expression. The line HTB 21 was found to endogenously express B1501. To determine if clone 1-9A would recognize the tumor cell line HTB21, an IFN-gamma ELISPOT assay was performed using 20,000 T cells, low dose IL-2 (5 ug/ml), and 20,000 of the following targets: autologous B726P or Mart-1 fibroblasts, untransduced

or B1501-transduced HTB21; or untransduced or B1501-transduced HTB132. These were incubated overnight and the assay was developed the next day. The results of this assay are shown in Figure 2. These studies demonstrate that B726P-specific CTL can recognize and lyse breast tumor cells expressing B726P.

EXAMPLE 5

IDENTIFICATION OF IMMUNOGENIC CD4 T CELL EPITOPES IN BREAST ANTIGENS

Immunogenic CD4 T cell epitopes derived from the breast antigen B726P were identified as follows.

A total of thirty-five 20-mer peptides overlapping by 12 amino acids and derived from the downstream ORF of B726P (corresponding to amino acids 1-317 of SEQ ID NO:176) were generated by standard procedure. Dendritic cells (DC) were derived from PBMC of a normal male donor using GMCSF and IL-4 by standard protocol. Purified CD4 T cells were generated from the same donor as the DC using MACS beads and negative selection of PBMCs. DC were pulsed overnight with pools of the 20-mer peptides, with each peptide at an individual concentration of 0.5 micrograms/mL. Pulsed DC were washed and plated at 10,000 cells/well of 96-well U bottom plates, and purified CD4 T cells were added at 100,000 cells/well. Cultures were supplemented with 10 ng/mL IL-6 and 5 ng/mL IL-12 and incubated at 37 °C. Cultures were restimulated as above on a weekly basis using DC made and pulsed as above as the antigen presenting cell, supplemented with 10 u/mL IL-2 and 5 ng/mL IL-7. Following three *in vitro* stimulation cycles (the initial priming + two restimulations), cell lines (each corresponding to one well) were tested for specific proliferation and cytokine production in response to the stimulating pool versus an irrelevant pool of peptides derived from unrelated antigens. A number of individual CD4 T cell lines (36/672 by IFN-gamma and 64/672 by proliferation) demonstrated significant cytokine release (IFN-gamma) and proliferation in response to the B726P peptide pools but not to the control peptide pool. Twenty-five of these T cell lines were restimulated on the appropriate pool of B726P peptides and reassayed on autologous DC pulsed with either the individual peptides or recombinant B726P protein made in *E.*

coli. Approximately 14 immunogenic peptides were recognized by the T cells from the entire set of peptide antigens tested. The amino acid sequences of these 14 peptides are provided in SEQ ID NO:534 - 547, with the corresponding DNA sequences being provided in SEQ ID NO:520-533, respectively. In some cases the peptide reactivity of the T cell line could be mapped to a single peptide but some could be mapped to more than one peptide in each pool. Thirteen of the fifteen T cell lines recognized the recombinant B726P protein. These results demonstrate that 13 of the 14 peptide sequences (SEQ ID NO:534-542 and 544-547) may be naturally processed CD4 epitopes of the B726P protein.

EXAMPLE 6

PREPARATION AND CHARACTERIZATION OF ANTIBODIES AGAINST BREAST TUMOR ANTIGEN B726P

Polyclonal antibodies against both the downstream (SEQ ID NO:176) and upstream (SEQ ID NO:469) ORF of the breast tumor antigen B726P were prepared as follows.

The downstream or upstream ORF of B726P expressed in an *E. coli* recombinant expression system was grown overnight in LB broth with the appropriate antibiotics at 37 °C in a shaking incubator. The next morning, 10 ml of the overnight culture was added to 500 ml to 2x YT plus appropriate antibiotics in a 2L-baffled Erlenmeyer flask. When the Optical Density (at 560 nm) of the culture reached 0.4-0.6, the cells were induced with IPTG (1 mM). Four hours after induction with IPTG, the cells were harvested by centrifugation. The cells were then washed with phosphate buffered saline and centrifuged again. The supernatant was discarded and the cells were either frozen for future use or immediately processed. Twenty ml of lysis buffer was added to the cell pellets and vortexed. To break open the *E. coli* cells, this mixture was then run through the French Press at a pressure of 16,000 psi. The cells were then centrifuged again and the supernatant and pellet were checked by SDS-PAGE for the partitioning of the recombinant protein. For proteins that localized to the cell pellet, the pellet was resuspended in 10 mM Tris pH 8.0, 1% CHAPS and the inclusion body pellet was washed and centrifuged again.

This procedure was repeated twice more. The washed inclusion body pellet was solubilized with either 8 M urea or 6 M guanidine HCl containing 10 mM Tris pH 8.0 plus 10 mM imidazole. The solubilized protein was added to 5 ml of nickel-chelate resin (Qiagen) and incubated for 45 min to 1 hour at room temperature with continuous agitation. After incubation, the resin and protein mixture were poured through a disposable column and the flow through was collected. The column was then washed with 10-20 column volumes of the solubilization buffer. The antigen was then eluted from the column using 8M urea, 10 mM Tris pH 8.0 and 300 mM imidazole and collected in 3 ml fractions. A SDS-PAGE gel was run to determine which fractions to pool for further purification.

As a final purification step, a strong anion exchange resin, such as HiPrepQ (Biorad), was equilibrated with the appropriate buffer and the pooled fractions from above were loaded onto the column. Antigen was eluted off the column with a increasing salt gradient. Fractions were collected as the column was run and another SDS-PAGE gel was run to determine which fractions from the column to pool. The pooled fractions were dialyzed against 10 mM Tris pH 8.0. The protein was then vialed after filtration through a 0.22 micron filter and the antigens were frozen until needed for immunization.

Four hundred micrograms of the B726P antigen was combined with 100 micrograms of muramyldipeptide (MDP). Every four weeks rabbits were boosted with 100 micrograms mixed with an equal volume of Incomplete Freund's Adjuvant (IFA). Seven days following each boost, the animal was bled. Sera was generated by incubating the blood at 4 °C for 12-24 hours followed by centrifugation.

Ninety-six well plates were coated with B726P antigen by incubating with 50 microliters (typically 1 microgram) of recombinant protein at 4 °C for 20 hours. 250 Microliters of BSA blocking buffer was added to the wells and incubated at room temperature for 2 hours. Plates were washed 6 times with PBS/0.01% Tween. Rabbit sera was diluted in PBS. Fifty microliters of diluted sera was added to each well and incubated at room temperature for 30 min. Plates were washed as described above before 50 microliters of goat anti-rabbit horse radish peroxidase (HRP) at a 1:10000 dilution was added and incubated at room temperature for 30 min. Plates were again washed as

described above and 100 microliters of TMB microwell peroxidase substrate was added to each well. Following a 15 min incubation in the dark at room temperature, the colorimetric reaction was stopped with 100 microliters of 1N H_2SO_4 and read immediately at 450 nm. All the polyclonal antibodies showed immunoreactivity to the appropriate B726P antigen.

B) Preparation of polyclonal antibodies against B709P and B720P

The breast antigens B709P (SEQ ID NO: 62) and B720P (SEQ ID NO: 485) expressed in an *E. coli* recombinant expression system were grown overnight in LB Broth with the appropriate antibiotics at 37°C in a shaking incubator. Ten ml of the overnight culture was added to 500 ml of 2x YT plus appropriate antibiotics in a 2L-baffled Erlenmeyer flask. When the optical density (at 560 nanometers) of the culture reached 0.4-0.6, the cells were induced with IPTG (1 mM). Four hours after induction with IPTG, the cells were harvested by centrifugation. The cells were washed with phosphate buffered saline and centrifuged again. The supernatant was discarded and the cells were either frozen for future use or immediately processed. Twenty milliliters of lysis buffer was added to the cell pellets and vortexed. To break open the *E. coli* cells, the mixture was run through a French Press at a pressure of 16,000 psi. The cells were centrifuged again and the supernatant and pellet were checked by SDS-PAGE for the partitioning of the recombinant protein. For proteins that localized to the cell pellet, the pellet was resuspended in 10 mM Tris pH 8.0, 1% CHAPS and the inclusion body pellet was washed and centrifuged again. This procedure was repeated twice more. The washed inclusion body pellet was solubilized with either 8 M urea or 6 M guanidine HCl containing 10 mM Tris pH 8.0 plus 10 mM imidazole. The solubilized protein was added to 5 ml of nickel-chelate resin (Qiagen) and incubated for 45 min to 1 hour at room temperature (RT) with continuous agitation. After incubation, the resin and protein mixture were poured through a disposable column and the flow through was collected. The column was then washed with 10-20 column volumes of the solubilization buffer. The antigen was then eluted from the column using 8M urea, 10 mM Tris pH 8.0 and 300 mM imidazole and collected in 3 ml fractions. A SDS-PAGE gel was run to determine which fractions to pool for further

purification. As a final purification step, a strong anion exchange resin such as Hi-Prep Q (Biorad) was equilibrated with the appropriate buffer and the pooled fractions from above were loaded onto the column. Each antigen was eluted off of the column with an increasing salt gradient. Fractions were collected as the column was run and another SDS-PAGE gel was run to determine which fractions from the column to pool. The pooled fractions were dialyzed against 10 mM Tris pH 8.0. The proteins were then vialed after filtration through a 0.22-micron filter and frozen until needed for immunization.

Four hundred micrograms of antigen was combined with 100 micrograms of muramyldipeptide (MDP). An equal volume of Incomplete Freund's Adjuvant (IFA) was added and mixed, and the mixture was injected into a rabbit. The rabbit was boosted with 100 micrograms of antigen mixed with an equal volume of IFA every four weeks. The animal was bled seven days following each boost. Sera was generated by incubating the blood at 4°C for 12-24 hours followed by centrifugation.

The reactivity of the polyclonal antibodies to recombinant antigen (B709P or B720P) was determined by ELISA as follows. Ninety-six well plates were coated with antigen by incubating with 50 microliters (typically 1 microgram) at 4°C for 20 hrs. 250 microliters of BSA blocking buffer was added to the wells and incubated at RT for 2 hrs. Plates were washed 6 times with PBS/0.01% Tween. Rabbit sera were diluted in PBS. Fifty microliters of diluted sera was added to each well and incubated at RT for 30 min. Plates were washed as described above before 50 microliters of goat anti-rabbit horse radish peroxidase (HRP) at a 1:10000 dilution was added and incubated at RT for 30 min. Plates were washed as described above and 100 microliters of TMB Microwell Peroxidase Substrate was added to each well. Following a 15-minute incubation in the dark at RT, the colorimetric reaction was stopped with 100 microliters of 1N H_2SO_4 and read immediately at 450 nm. The polyclonal antibodies showed immunoreactivity to the appropriate antigen.

EXAMPLE 7

PROTEIN EXPRESSION OF BREAST TUMOR ANTIGENS

The downstream ORF of B726P (SEQ ID NO:176), together with a C-terminal 6X His Tag, was expressed in insect cells using the baculovirus expression system as follows.

The cDNA for the full-length downstream ORF of B726P was PCR amplified using the primers of SEQ ID NO:480 and 481. The PCR product with the expected size was recovered from agarose gel, restriction digested with EcoRI and Hind II, and ligated into the transfer plasmid pFastBac1, which was digested with the same restriction enzymes. The sequence of the insert was confirmed by DNA sequencing. The recombinant transfer plasmid pFBB726P was used to make recombinant bacmid DNA and virus using the Bac-To-Bac Baculovirus expression system (BRL Life Technologies, Gaithersburg, MD). High Five cells were infected with the recombinant virus BVB726P to produce protein. The cDNA and amino acid sequences of the expressed B726P recombinant protein are provided in SEQ ID NO:482 and 483, respectively.

EXAMPLE 8

GENERATION OF CONSTRUCTS FOR PROTEIN EXPRESSION OF B726P IN *E. COLI*

Three different open reading frames (ORFs) of B726P were subcloned into pPDM, a modified pET28 vector for exression in *E. coli*.

Construct for the expression of B726P Upstream ORF in *E. coli* (cDNA: SEQ ID NO:549; amino acid: SEQ ID NO:552):

The partial B726P upstream ORF (A) from clone 23113 was PCR amplified with the following primers:

PDM-416 (SEQ ID NO:554) 5' gtcggctccatgagtcccgcaaaag 3' Tm 63^{O}C.

PDM-431 (SEQ ID NO:555) 5' cgagaattcaata**cttaag**aagaccatctttaccag 3' Tm 61^{O}C.

The amplification conditions were as follows 10 µl 10X Pfu buffer, 1 µl 10 µM dNTPs, 2 µl 10 µM each oligo, 83 µl sterile water, 1.5 µl Pfu DNA polymerase

(Stratagene, La Jolla, CA), 1 µl PCR 23113. The reaction was first denatured for 2 minutes at 96°C, followed by 40 cylces of 96°C for 20 seconds, 62°C for 15 seconds, and extension at 72°C for 2 minutes. This was followed by a final extension of 72°C for 4 minutes.

The second partial B726P upstream ORF (B) from clone 19310 was PCR amplified with the following primers:

PDM-432 (SEQ ID NO:556) 5' cataag**cttaag**gctaactgcggaatgaaag 3' Tm 63°C.

PDM-427 (SEQ ID NO:557) 5' cccgcagaattcaacatgcaattttcatgtaagag 3' Tm 62°C.

The amplification and cycling conditions were as described above. The first PCR product was digested with EcoRI and cloned into pPDM His (a modified pET28 vector) that had been digested with EcoRI and Eco72I. The second PCR product was digested with BfrI and EcoRI and cloned into the resulting construct: pPDM B726P UP-A-5 at the EcoRI and BfrI sites. The construct (pPDM B726P Up-4) was confirmed to be correct through sequence analysis and transformed into BL21 (DE3) pLys S and BL21 CodonPlus RIL (DE3) cells. Protein expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

Construct for B726P D-ORF expression in *E. coli* (cDNA: SEQ ID NO:550; amino acid: SEQ ID NO:551):

The B726P D-ORF was PCR amplified with the following primers:

PDM-290 (SEQ ID NO:558) 5' ctaaatgccggcacaagagctctgc 3' Tm 61°C

PDM-291 (SEQ ID NO:559) 5' cgcgcagaattctattatataacttctgtttctgc 3' Tm61°C

The reaction conditions were as described. The cycling conditions were altered slightly in that the annealing temperature was lowered to 61°C from 62°C and was held for 15 seconds. The extension time was also increased to 2 minutes and 15 seconds. The PCR product was digested with NaeI and EcoRI and cloned into pPDM His which has been digested with Eco72I and EcoRI. Construct was confirmed by sequencing and then transformed into BL21 (DE3) pLys S cells (Novagen, Madison, WI). Protein expression

was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

Construct for B726P Combined ORF expression in *E. coli* (cDNA: SEQ ID NO:548; amino acid: SEQ ID NO:553):

The B726P C-1 coding region was PCR amplified including the 183bp insert, with the following primers:

PDM-750 (SEQ ID NO:560) 5' ggggaattgtgagcggataacaattc 3' Tm 58°C

PDM-752 (SEQ ID NO:561) 5' cgtagaattcaacctgatttaaattactttctacac 3' Tm 59°C

The B726P Downstream ORF was PCR amplified with the following primers:

PDM-753 (SEQ ID NO:562) 5' gaaagtaatttaaatcaggtttctcacactc 3' Tm 59°C

PDM-751 (SEQ ID NO:563) 5' gaggccccaaggggttatgctag 3' Tm 61°C

The reaction conditions for these PCR reactions were the same as described above. The cycling conditions were as follows: 1st PCR: The reaction was first denatured for 2 minutes at 96°C, followed by 40 cylces of 96°C for 20 seconds, 58°C for 15 seconds, and extension at 72°C for 4 minutes. This was followed by a final extension of 72°C for 4 minutes; 2nd PCR: . The reaction was first denatured for 2 minutes at 96°C, followed by 40 cylces of 96°C for 20 seconds, 59°C for 15 seconds, and extension at 72°C for 2 minutes. This was followed by a final extension of 72°C for 4 minutes. The first PCR product was digested with EcoRI and cloned into pPDM His (a modified pET28 vector) at the Eco 72I and EcoRI sites. The construct was confirmed to be correct through sequence analysis. The second PCR product was digested with EcoRI and cloned into pPDM His at the same sites. The resulting constructs pPDM B726P UA-8 and pPDM B726P DA-7 respectively were digested with SwaI and EcoRI. The pPDM B726P UA-8 construct was used as vector and the insert from the pPDM B726P DA-7 was cloned into this construct successfully. The construct was confirmed to be correct through sequence analysis and then transformed into BLR (DE3) pLys S and HMS 174 (DE3) pLys S cells (Novagen, Madison, WI). Protein

expression was confirmed by Coomassie stained SDS-PAGE and N-terminal protein sequence analysis.

EXAMPLE 9

ADDITIONAL SEQUENCE IDENTIFIED FOR BREAST TUMOR ANTIGEN B726P BY BIOINFORMATIC ANALYSIS

The combined ORF of the breast tumor antigen, B726P (amino acid sequence set forth in SEQ ID NO:475), was used to search public databases. A sequence essentially identical to the combined ORF with additional N-terminal sequence was identified in the GenBank nonredundant protein database and the cDNA and predicted amino acid sequences are set forth in SEQ ID NO:564 and 565, respectively. The gene is also referred to as NY-BR-1 and was described in described in Cancer Research 61(5):2055-2061, March 1, 2001.

EXAMPLE 10

ANALYSIS OF B726P EXPRESSION USING IMMUNOHISTOCHEMISTRY

Affinity purified polyclonal antibodies anti-B726Pup (generated against the B726P upstream ORF protein) and anti-B726Pdown (generated against the B726P downstream ORF) were used to assess B726P protein expression in breast cancer and in a variety of normal tissue sections.

In order to determine which tissues express the breast cancer antigen protein B726P immunohistochemistry (IHC) analysis was performed on a diverse range of tissue sections. Tissue samples were fixed in formalin solution for 12-24 hrs and embedded in paraffin before being sliced into 8 micron sections. Steam heat induced epitope retrieval (SHIER) in 0.1 M sodium citrate buffer (pH 6.0) was used for optimal staining conditions. Sections were incubated with 10% serum/PBS for 5 minutes. Primary antibody (either rabbit affinity purified anti-B726Pdown or anti-B726Pup) was added to each section for 25 minutes followed by 25 minute incubation with anti-rabbit biotinylated antibody. Endogenous peroxidase activity was blocked by three 1.5 minute incubations with

hydrogen peroxidase. The avidin biotin complex/horse radish peroxidase (ABC/HRP) system was used along with DAB chromogen to visualize antigen expression. Slides were counterstainied with hematoxylin to visualize cell nuclei. Anti-B726Pup and anti-B726Pdown immunoreactivity was observed in about 30-40% of breast cancer samples analyzed but not observed in a majority of various normal tissues. Anti-B726Pdown and anti-b726Pup also stained roughly the same breast cancer samples. Thus, these data confirm earlier microarray analysis (see Example 1) showing that B726P is overexpressed in breast tumor tissue as compared to normal tissue. Therefore, this antigen may be used in diagnostic and immunotherapeutic applications for breast cancer.

EXAMPLE 11

GENERATION OF MONOCLONAL ANTIBODIES TO B726P DOWNSTREAM AND UPSTREAM ORFS

Production and purification of protein used for antibody generation. B726 upstream ORF and B726 downstream ORF proteins were expressed in an *E. coli* recombinant expression system (see Example 8). Cells were grown overnight in LB Broth with the appropriate antibiotics at 37°C in a shaking incubator. The next morning, 10 ml of the overnight culture was added to 500 ml of 2x YT plus appropriate antibiotics in a 2L-baffled Erlenmeyer flask. When the optical density (at 560 nanometers) of the culture reached 0.4-0.6 the cells were induced with IPTG (1 mM). Four hours after induction with IPTG the cells were harvested by centrifugation. The cells were then washed with phosphate buffered saline and centrifuged again. The supernatant was discarded and the cells were either frozen for future use or immediately processed. Twenty milliliters of lysis buffer was added to the cell pellets and vortexed. To break open the *E. coli* cells, this mixture was then run through the French Press at a pressure of 16,000 psi. The cells were then centrifuged again and the supernatant and pellet were checked by SDS-PAGE for the partitioning of the recombinant protein. For proteins that localized to the cell pellet, the pellet was resuspended in 10 mM Tris pH 8.0, 1% CHAPS and the inclusion body pellet was washed and centrifuged again. This procedure was repeated twice more. The washed

inclusion body pellet was solubilized with either 8 M urea or 6 M guanidine HCl containing 10 mM Tris pH 8.0 plus 10 mM imidazole.

The solubilized protein was added to 5 ml of nickel-chelate resin (Qiagen, Valencia, CA) and incubated for 45 min to 1 hour at room temperature with continuous agitation. After incubation, the resin and protein mixture were poured through a disposable column and the flow through was collected. The column was then washed with 10-20 column volumes of the solubilization buffer. The antigen was then eluted from the column using 8M urea, 10 mM Tris pH 8.0 and 300 mM imidazole and collected in 3 ml fractions. A SDS-PAGE gel was run to determine which fractions to pool for further purification. As a final purification step, a strong anion exchange resin such as Hi-Prep Q (Biorad) was equilibrated with the appropriate buffer and the pooled fractions from above were loaded onto the column. Each antigen was eluted off of the column with an increasing salt gradient. Fractions were collected as the column was run and another SDS-PAGE gel was run to determine which fractions from the column to pool. The pooled fractions were dialyzed against 10 mM Tris pH 8.0. This material was then submitted to Quality Control for final release. The release criteria were purity as determined by SDS-PAGE or HPLC, concentration as determined by Lowry assay or Amino Acid Analysis, identity as determined by amino terminal protein sequence, and endotoxin level as determined by the Limulus (LAL) assay. The proteins were then vialed after filtration through a 0.22-micron filter and the antigens were frozen until needed for immunization.

To generate anti-B726P mouse monoclonal antibodies, mice were immunized IP with 50 micrograms of recombinant B726P upstream ORF and B726P downstream ORF proteins that had been mixed to form an emulsion with an equal volume of Complete Freund's Adjuvant (CFA). Every three weeks animals were injected IP with 50 micrograms of recombinant B726P upstream ORF and B726P downstream ORF that had been mixed with an equal volume of IFA to form an emulsion. After the fourth injection, spleens were isolated and standard hybridoma fusion procedures were used to generate anti-B726P mouse monoclonal antibody hybridomas. Anti-B726P monoclonal

antibodies were screened using the ELISA analysis using the bacterially expressed recombinant B726P upstream ORF and B726P downstream ORF proteins.

A list of the mouse anti-B726P monoclonal antibodies that were generated, as well as their anti-B726P reactivity in an ELISA assay and Western blot are shown in Table 2. The hybridomas were then subcloned and the subclones further tested for reactivity with B726P upstream ORF and B726P downstream ORF proteins. Several monoclonal antibodies showed particularly favorable reactivity: 220A2-21, 220A19-25, 220A94-29, 220A151-33.

For Western blot analysis, recombinant B726P upstream ORF and B726P downstream ORF protein was diluted with SDS-PAGE loading buffer containing beta-mercaptoethanol, then boiled for 10 minutes prior to loading the SDS-PAGE gel. Protein was transferred to nitrocellulose and probed with each of the anti-B726P hybridoma supernatants. Protein A-HRP was used to visualize the anti-B726P reactive bands by incubation in ECL substrate.

TABLE 2: B726PUP AND B726PDOWN MONOCLONAL ANTIBODY REACTIVITY

	ELISA			Western Blots	
Anti-B726P mAbs	B726PDown	B726PUp	L523S	B726Pdown	B726Pup
220A2	+++	+	-	+++	++
220A10	-	-	-	N/A	N/A
220A14	+++	+++	+++	+++	++
220A19	++	-	-	++	+
220A43	+++	+	-	+++	++
220A86	+++	+	-	+++	++
220A94	+++	-	-	++	+/-
220A123	++	-	-	+	-
220A139	+/-	-	-	+	-
220A140	-	-	-	N/A	N/A

220A141	-	-	-	N/A	N/A
220A143	-	-	-	N/A	N/A
220A151	++	-	-	++	-
220A176	+/-	-	-	+	-

EXAMPLE 12

IDENTIFICATION OF ADDITIONAL SEQUENCES FOR B726P

Additional 5' sequence was obtained for B726P - this sequence was obtained by PCR from 1st strand cDNA prepared from three separate mRNA sources (metastatic breast tumor, breast tumor, normal testis). Disclosed herein are clones that were isolated, each with differences from the expected published sequence of NY-BR-1.

A 1300 bp fragment of B726P otherwise known as NY-BR-1 was PCR amplified from 1st strand cDNA and cloned into pPDM, a modified pET28 vector as follows:

The B726P XB coding region (NY-BR-1) was PCR amplified with the following primers

PDM-784 5' cacacaaagaggaagaagaccatc 3' Tm 56°C

PDM-814 5' gattcttttgtaggacatgcaatcatc 3' Tm55°C

The following PCR conditions were used: 10µl 10X Herculase buffer, 1µl 10mM dNTPs, 2µl 10.µM each oligo, 83µl sterile water, 1.5µl Herculase DNA polymerase (Stratagene, La Jolla, CA), 50 ng DNA. The thermalcycling conditions were as follows:

98°C 3 minutes

98°C 40 seconds, 51°C 15 seconds, 72°C 4 minutes, X 10 cycles

98°C 40 seconds, 51°C 15 seconds, 72°C 5 minutes, X 10 cycles

98°C 40 seconds, 51°C 15 seconds, 72°C 6 minutes, X 10 cycles

98°C 40 seconds, 51°C 15 seconds, 72°C 7 minutes, X 10 cycles

72°C 10 minutes

The PCR product was ligated into the pPDM vector (a modified pET28) that had been digested with Eco72I and de-phosphorylated. PCR amplification of this gene proved very difficult and required the use of a polymerase lacking proofreading capabilities. However, use of such an enzyme, in this case, Herculase from Stratagene (La Jolla, CA), led to what is likely PCR errors in the resulting clones. The cDNA sequence of three of the isolated clones containing mutations are disclosed in SEQ ID NO:567-569 with the corresponding amino acid sequences disclosed in SEQ ID NO:572, 571, and 570, respectively.

The resulting construct, pPDM B726P XB (clone 83686), was then digested with BglII and the insert which dropped out from the 5' vector BglII site and the internal BglII site at amino acids 390-391 was cloned into the pPDM B726P C-ORF (SEQ ID NO:548) that had been digested with BglII and was de-phosphorylated. This construct, pPDM B726P XC, was then DNA sequenced and showed two nucleotide changes, which result in two amino acid changes. The cDNA of the full-length clone containing these 2 mutations is disclosed in SEQ ID NO:566 with the corresponding amino acid sequence in SEQ ID NO:573. The full-length expected, published NY-BR-1 is disclosed in SEQ ID NO:564 (cDNA); amino acid SEQ ID NO:565.

EXAMPLE 13

ISOLATION OF ADDITIONAL 3' SEQUENCE AND REAL-TIME PCR ANALYSIS OF B726P HOMOLOG NY-BR1.1

A sequence homolog to the breast candidate B726P, called NY-BR-1.1, was identified and published in Cancer Research 61(5):2055-2061; March 1, 2001. The NY-BR-1.1 gene, thought to be located on chomosome 9 based on 100% sequence identity to genomic sequence from chromosome 9, was shown to be expressed as mRNA in breast tumors as well as in normal brain. However, the published sequence was lacking 3' sequence. Published incomplete sequence for NY-BR-1.1 is represented by GenBank accession number AF269088. A recent BlastN search of the GenBank High Throughput

Genomic Sequence database using Ny-Br-1.1 as a query sequence showed a 100% match to the working draft sequence of chromosome 9 (GenBank accession number AL359312), yielding further 3' DNA sequence for Ny-Br-1.1. The compilation of the Ny-Br-1.1 sequence with the additional 3' sequence from chromosome 9 yielded a 3720 bp ORF sequence (SEQ ID NO:576) which encodes a 1240 amino acid protein sequence (SEQ ID NO:577).

Real time PCR primers were designed to a unique region of NY-BR-1.1 to distinguish its mRNA expression profile from B726P. This experiment represents relative values, as it was done without template. The first-strand cDNA used in the quantitative real-time PCR was synthesized from 20 μg of total RNA that was treated with DNase I (Amplification Grade, Gibco BRL Life Technology, Gaithersburg, MD), using Superscript Reverse Transcriptase (RT) (Gibco BRL Life Technology, Gaithersburg, MD). Real-time PCR was performed with a GeneAmp™ 5700 sequence detection system (PE Biosystems, Foster City, CA). The 5700 system uses SYBR™ green, a fluorescent dye that only intercalates into double stranded DNA, and a set of gene-specific forward and reverse primers. The increase in fluorescence was monitored during the whole amplification process. The optimal concentration of primers was determined using a checkerboard approach and a pool of cDNAs from tumors was used in this process. The PCR reaction was performed in 25 μl volumes that included 2.5 μl of SYBR green buffer, 2 μl of cDNA template and 2.5 μl each of the forward and reverse primers for the gene of interest. The cDNAs used for quantitative real time PCR reactions were diluted 1:10 for each gene of interest and 1:100 for the β-actin control. Levels of mRNA were expressed relative to ureter where NY-BR-1.1 expression was not observed when compared to the β-actin control.

The real time PCR results show that mRNA expression for NY-BR-1.1 is present in breast tumors as well as in normal adrenal gland, brain, retina and testis.

EXAMPLE 14

CHARACTERIZATION OF B726P MONOCLONAL AND PURIFIED POLYCLONAL ANTIBODY EPITOPES

Mouse monoclonal antibodies and rabbit polyclonal sera were raised against *E. coli* derived B726P recombinant protein and tested by ELISA as described in further detail below, for antibody epitope recognition against overlapping 20 mer peptides that correspond to the amino acid sequence of the downstream ORF of B726P (B726P dORF, set forth in SEQ ID NO:176, encoded by SEQ ID NO:175). Numerous peptides were recognized by the monoclonal and polyclonal antibodies. The corresponding amino acid sequences of these peptide antibody epitopes are summarized in Table 3 and are set forth in SEQ ID NO:578-593.

ELISA ANALYSIS: B726P recombinant protein and peptides were coated onto 96 well ELISA plate: 50ul/well at 2 ug/ml for 20 hrs at 4C. Plates were then washed 5 times with PBS + 0.1% Tween 20 and blocked with PBS + 1% BSA for 2 hr. Affinity purified B726P polyclonal antibodies were then added to the wells at 1ug/ml and B726P monoclonal supernatants were added neat (220A43 and 220A86 were diluted 1/60 and 1/20 respectively). Plates were incubated at room temperature for 30 minutes and then washed again as above, followed by the addition of 50ul/well donkey anti-mouse-Ig-HRP antibody for 30 minutes at room temperature. Plates were washed, then developed by the addition of 100ul/well of TMB substrate. The reaction was incubated 15 minutes in the dark at room temperature and then stopped by the addition of 100ul/well of 1N H2SO4. Plates were read at OD450 in an automated plate reader. Peptides with OD450 readings three times background or above were considered to be positively recognized by the corresponding antibody.

Table 3:Peptides recognized by B726P Antibodies

	B726P Monoclonal Supernatant						B726P Purified Polyclonal
	220A2.1	220A19.1	220A94.1	220A151.1	220A43	220A86	(1ug/ml)
B726P peptides (amino acids)	289-308	225-244, 232-252	73-252	145-164, 153-172	232-252	145-164, 153-172	1-20, 9-28, 17-36, 24-44, 97-116, 105-124, 113-132, 121-140, 129-148, 137-156

EXAMPLE 15

ANALYSIS OF AUTOANTIBODIES TO B726P IN BREAST CANCER SERA AND EPITOPE MAPPING OF THE ANTIGENIC SITES

Specific B726P peptide epitopes were identified that react with autoantibodies in the serum of breast cancer patients. Thirty-three overlapping peptides were synthesized spanning the entire B726P protein. These 33 peptides were tested in ELISAs to evaluate which epitopes reacted with breast cancer sera. Reactive epitopes were identified throughout the molecule and a total of 16/74 sera samples from breast cancer patients had reactivity with one or more peptides.

Thirty-one overlapping synthetic peptides spanning the entire B726P downstream ORF sequence (amino acid sequence set forth in SEQ ID NO:176) were synthesized and 30 of these were tested in ELISA with sera from breast cancer patients as well as control sera. The amino acid sequences of the 31 overlapping peptides of the B726P downstream ORF are set forth in SEQ ID NO:594-624. Three additional peptides of B726P, set forth in SEQ ID NO:625-627 were also tested. Several peptides thoughout the molecule showed reactivity, in particular peptide #2735 (amino acids 31-50; SEQ ID NO:597), peptide #2747 (amino acids 151-170; SEQ ID NO:609), peptide #2750 (amino acids 181-200; SEQ ID NO:612), peptide #2753 (amino acids 211-230; SEQ ID NO:615), and peptide #2766 (amino acids 231-250; SEQ ID NO:617). A total of 16/74 breast cancer sera were reactive with at least one peptide.

B726P antibody epitopes were also mapped using rabbit antisera generated against the B726P downstream ORF (SEQ ID NO:176). The epitopes identified using the rabbit antisera were as follows: peptide #2732 (amino acids 1-20; SEQ ID NO:594), peptide # 2733 (amino acids 11-30; SEQ ID NO:595), peptide #2742 (amino acids 101-120; SEQ ID NO:604), peptide #2743 (amino acids 111-130; SEQ ID NO:605), peptide #2744 (amino acids 121-140; SEQ ID NO:606), peptide #2745 (amino acids 130-151; SEQ ID NO:607), peptide #2751 (amino acids 191-210; SEQ ID NO:613), and peptide #2753 (amino acid 211-230; SEQ ID NO:615). Some low level reactivity was observed for

peptide #2772 (amino acids 291-310; SEQ ID NO:623) and peptide #2773 (amino acids 298-317; SEQ ID NO:624).

The above results confirm that B726P can be used alone or in combination wtih other breast tumor antigens as a vaccine target. Additionally, these results show that detection of antibodies to B726P can be used as a diagnostic indicator of breast cancer either alone or in combination with detection of antibodies to other antigens (*e.g.* Her-2/Neu or other antigens known to be expressed in breast cancer tissue).

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.